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Expression of RUNX2 and MDM21 in rats with periodontitis under chronic intermittent hypoxia

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ABSTRACT

Objective: To discuss the expression of RUNX2 and MDM21 in rats with periodontitis under the chronic intermittent hypoxia.

Methods: A total of 32 SD healthy rats were randomly divided into four groups, with 8 rats in each group. The molecular biological techniques of immunohistochemistry, RT-PCR and Western blotting were employed to detect the effect of different hypoxia time (0, 6, 12, 24 and 48 h) and different concentrations of hypoxia (0.000, 0.001, 0.010, 0.060 and 0.100 ppm) on the expression of RUNX2 and MDM21 in rats of four groups.

Results: The expression of RUNX2 and MDM21 in each group was significantly higher than the one at other concentrations when the concentration was 0.010 ppm, with the statistical difference ($P < 0.05$). The expression of RUNX2 and MDM21 was that normoxic control group > normoxic periodontitis group > hypoxia control group > hypoxia periodontitis group under the action with the concentration of 0.010 ppm for 12 h, but there was no significant difference for the comparison among groups ($P > 0.05$).

Conclusions: The condition of chronic intermittent hypoxia can reduce the expression of RUNX2 and MDM21 in rats with periodontitis and aggravate the damage of periodontal bone.

1. Introduction

The periodontitis is some kind of chronic infectious diseases in the periodontal tissue, as one of two major oral diseases. Its prevalence was high around the world, where the most common one was chronic periodontitis, occupying about 90% of total cases of periodontal diseases [1–3]. Such disease may appear among all age groups, but it is more common to find it in adults. The clinical researches [4] indicated that the periodontitis could not only have the severe impact on the oral health, but also affect the occurrence and development of many systemic diseases. The obstructive sleep apnea-hypopnea syndrome (OSAHS) is a common clinical disease and it involves many disciplines, with the main manifestations of breathing disorder and sleep disorder. Its pathological changes include the frequent alternation of chronic intermittent hypoxia and reaeration and then the hypoxia/reoxygenation causes the systematic and local inflammatory response. The previous researches also proved that the periodontitis and OSAHS were all diseases related to the inflammatory response that affected the immune response and its reason was related to the invasion of periodontopathic microorganisms and its toxic products [5–8]. But it's unknown whether it further affected the periodontal bone. In this study, based on the rat model of chronic intermittent hypoxiaperiodontitis, it was to observe its effect on the expression of osteogenic markers RUNX2 and MDM21 in the condition of periodontitis and OSAHS and discuss the effect of periodontitis on the damage of dental bone tissues of rats in the condition of chronic intermittent hypoxia.

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2. Materials and methods

2.1. Reagents, animals and instruments

2.1.1. Reagents

200 g SD rats were provided by Laboratory Animal Center, Zhengzhou University. The study protocol, operation procedure and animal ethics were all approved by School of Medical Sciences, Zhengzhou University. The immunohistochemistry kit was purchase from GIBCO, PCR kit from Shanghai Solomon Biotechnology Co., Ltd., chloral hydrate from Wuhan Boster, EDTA containing pancreatin from GIBCO, HRP labeled goat anti-rabbit IgG and HRP labeled goat anti-mouse IgG Shanghai Beyotime Biotechnology, primary antibody β-actin mouse polyclonal IgG and RUNX2 and MDM21 rabbit monoclonal IgG from Santa Cruz.

2.1.2. Instruments

PCR instrument was purchased from Rotor-gene, the trans-blot and electrophoresis apparatus from Bio-Rad Laboratories, the high speed refrigerated centrifuge BiofugeStratos, the inverted phase contrast microscope from Olympus, the autoclave from Tuttnauer, and −80 °C to 4 °C refrigerator from Panasonic.

2.2. Modeling and grouping

Eight of thirty-two rats were randomly chosen as the normoxic control group (group A) without the modeling. Rats in the normoxic periodontitis group (group B) received the operation for the modeling. Rats were given the intraperitoneal injection of 50 000 U penicillin 30 min before the operation to avoid the infection. The intraperitoneal injection of 10% (0.33 mL/100 g) chloral hydrate was adopted in rats for the anesthesia. Taking the lateral position and after the skin disinfection by Anerdian, 0.5 mm orthodontic ligature was applied to maxillary tooth neck of second molar of rats on both sides, making the ligature in the gingival sulcus without the damage against the gums as far as possible. Anerdian was also used after the operation, with the normal saline washing the wound. The incision was closed layer by layer. The above procedure should be operated in the sterile environment strictly. After the operation, rats were given the high-carbohydrate diet every day (100 g diet included 28 g whole milk powder, 56 g sucrose, 3 g fresh vegetables, 6 g whole wheat flour, 1 g liver powder, 4 g yeast powder and 2 g salt). The survival situation of rats was observed every day, including the mental state, temperature, water feeding and response to the external stimulus. Rats in the hypoxic control group (group C) were put in the environment of severe OSAS and hypoxia chamber, with the different time of hypoxia effect (0, 6, 12, 24 and 48 h) and different hypoxia concentrations (0.000, 0.001, 0.010, 0.060 and 0.100 ppm). The modeled rats with the periodontitis in the hypoxic periodontitis group were put in the environment of severe OSAS and hypoxia chamber, with the different time of hypoxia effect (0, 6, 12, 24 and 48 h) and different hypoxia concentrations (0.000, 0.001, 0.010, 0.060 and 0.100 ppm).

2.3. Immunohistochemistry assay

SP method was employed to detect the results of immunohistochemistry for rats in four groups. After the deparaffinage of tissue sections, they were washed with 0.01 mol/L PBS for 3 times and fixed with 40 g/L paraformaldehyde for 15 min. The endogenous peroxidase was removed with volume fraction 3% hydrogen peroxide for 15 min and then sealed with 50 μL non-immune serum. After adding 50 μL RUNX2 and MDM21 primary antibodies over night, it was taken out and washed with PBS the next day for 3 times. Afterwards, it was incubated with 50 μL biotinylated secondary antibody for 30 min and incubated with horseradish peroxidase for 30 min. It was then colored with DBA, redyed with hematoxylin and mounted with neutral balsam. Results: if the cell plasma and membrane shows brown or brownish yellow under the inverted microscope, it is the positive cell. The average optical density measurement of ImageJ was employed to measure the ratio of positive cells.

2.4. RT-PCR

The total RNA was extracted from 100 mg periodontal tissue of rats in four groups for RT-PCR detection, with the procedure as follows: 2 μg total RNA was collected from each group for cDNA reverse transcription and then 20 μL as the controlled reaction system. PCR included 18 s RNA, 2 μL reverse transcription products, RUNX2 and MDM21 primers. The whole reaction was performed according to RT-PCR procedure: the reaction condition of initial denaturation as 5 min and 95 °C and gradient denaturation of 94 °C, 60 °C, 72 °C and 72 °C for 30 s respectively, with 30 cycles in total. The agarose gel electrophoresis was performed on collected PCR products and then the electrophoresis image was photoed for the further gel imaging analysis. Taking 18sRNA reference as the control, the comparison analysis was taken on such imaging results and the quantified absorbance value (A value) was chosen as the final value.

2.5. Western blotting

The total protein was extracted from periodontal tissue of rats in four groups (100 mg in each group). SDS-PAGE gel electrophoresis was employed for the analysis and the semi-dry method for the membrane transfer. After half an hour of sealing by the skimmed milk powder, RUNX2 and MDM21 primary antibody was (1:500) was added for the incubation over night; after the membrane was washed with PBS the next day, the horseradish peroxidase labeled goat anti-rabbit secondary body (1:1 000) was added for the incubated for 30 min. After being washed with PBS, ECL photochemical method was employed for the development of protein bands, while color image analysis system for the absorbance analysis of protein.

2.6. Statistical analysis

SPSS21.0 was adopted for the analysis of all collected data. Data with the normal distribution was expressed by mean ± SD, following the normal distribution and homogeneity of variances. LSD test was employed for the comparison between groups and nonparametric test for the comparison of expression of RUNX2 and MDM21 mRNA and the expression of RUNX2 and MDM21 protein in different conditions of hypoxia with different concentrations and at different time points. Kruskal–Wallis H test was employed for the comparison among groups, while Mann–Whitney U test for the comparison between groups. $P < 0.05$ indicated the statistical difference.

3. Results

3.1. Immunohistochemistry to detect the expression of RUNX2 and MDM21 in three groups

Among four groups of periodontal tissue, the expression of RUNX2 and MDM21 was high in the normoxic control group, with the deep straining intensity. The expression of RUNX2 and MDM21 was decreased in turn in the normoxic periodontitis group, hypoxic control group and hypoxic periodontitis group, with the decreased staining intensity as well. The mean optical density in the normoxic periodontitis group, hypoxic control group and hypoxic periodontitis group was significantly lower than that in the normoxic control group, with the statistical difference ($P < 0.05$). But there was no significant difference between the normoxic periodontitis group, hypoxic control group and hypoxic periodontitis group ($P > 0.05$). Results of optical density by ImageJ were shown in Table 1.

3.2. Effect of chronic intermittent hypoxia on expression of RUNX2 and MDM21 mRNA in rats with periodontitis

3.2.1. Effect of different concentrations of hypoxia with the action time of 24 h on expression of RUNX2 and MDM21 mRNA

For the comparison of expression of RUNX2 and MDM21 mRNA in different groups at different concentrations of hypoxia, when the concentration was 0.010 ppm, the expression of RUNX2 and MDM21 was higher than other concentrations, with the statistical difference ($P < 0.05$), as shown in Table 2.

Table 1

Results of optical density by immunohistochemistry.

Optical density	Group A	Group B	Group C	Group D
RUNX2	2.58 ± 0.26	2.06 ± 0.22*	2.03 ± 0.17*	1.93 ± 0.13*
MDM21	2.49 ± 0.23	1.99 ± 0.20*	1.94 ± 0.17*	1.93 ± 0.13*

* Compared with the normoxic control group, $P < 0.05$.

3.2.2. Effect of 0.010 ppm hypoxia with different action time on expression of RUNX2 and MDM21 mRNA

In condition of 0.010 ppm hypoxia with the action time of 6, 12, 24 and 48 h, respectively, the expression of RUNX2 and MDM21 mRNA began to be increased at 6 h and reached the peak at 12 h. Under the action with the concentration of 0.010 ppm for 12 h, the expression of RUNX2 and MDM21 was that normoxic control group > periodontitis group > chronic intermittent hypoxia group > compound group, but there was no significant difference for the comparison among groups ($P > 0.05$), with results shown in Table 3.

3.3. Effect of chronic intermittent hypoxia on expression of RUNX2 and MDM21 protein in rats with periodontitis

3.3.1. Effect of different concentrations of hypoxia with the action time of 24 h on expression of RUNX2 and MDM21 protein

For the comparison of expression of RUNX2 and MDM21 at different concentrations of hypoxia, according to the results of PCR, when the concentration was 0.010 ppm, the expression of RUNX2 and MDM21 protein in three groups was higher than groups at other concentrations, with the statistical difference ($P < 0.05$), as shown in Table 4.

3.3.2. Effect of 0.010 ppm hypoxia with different action time on expression of RUNX2 and MDM21 protein

In condition of 0.010 ppm hypoxia with the action time of 6, 12, 24 and 48 h, respectively, the expression of RUNX2 and MDM21 protein began to be increased at 6 h and reached the peak at 12 h. Under the action with the concentration of 0.010 ppm for 12 h, the expression of RUNX2 and MDM21 was that normoxic control group > normoxic periodontitis group > hypoxia control group > hypoxia periodontitis group, but there was no significant difference for the comparison among groups ($P > 0.05$), with results shown in Table 5.

Table 2

Comparison of expression of RUNX2 and MDM21 mRNA at different concentrations of hypoxia.

Hypoxia concentration (ppm)	RUNX2 mRNA			MDM21 mRNA		
	Group B	Group C	Group D	Group B	Group C	Group D
0.000	0.82 ± 0.14*	0.72 ± 0.16*	0.72 ± 0.19*	0.89 ± 0.12*	0.85 ± 0.16*	0.79 ± 0.12*
0.001	1.15 ± 0.21*	1.24 ± 0.15*	1.08 ± 0.17*	1.28 ± 0.18*	1.39 ± 0.14*	1.08 ± 0.18*
0.010	1.72 ± 0.34	1.58 ± 0.44	1.52 ± 0.64	1.96 ± 0.66	1.88 ± 0.43	1.81 ± 0.65
0.060	1.18 ± 0.22*	1.22 ± 0.20*	1.05 ± 0.22*	1.20 ± 0.25*	1.29 ± 0.25*	1.05 ± 0.22*
0.100	0.68 ± 0.13*	0.87 ± 0.17*	0.98 ± 0.16*	0.69 ± 0.16*	0.88 ± 0.18*	0.99 ± 0.19*

* Compared with the concentration of 0.010, $P < 0.05$.

Table 3

Effect of 0.010 ppm hypoxia with different action time on expression of RUNX2 and MDM21 mRNA.

Action time of hypoxia concentration (h)	RUNX2 mRNA				MDM21 mRNA			
	Group A	Group B	Group C	Group D	Group A	Group B	Group C	Group D
0	0.88 ± 0.19*	0.76 ± 0.16*	0.72 ± 0.15*	0.73 ± 0.14*	0.92 ± 0.12*	0.79 ± 0.13*	0.80 ± 0.26*	0.79 ± 0.12*
6	1.37 ± 0.26*	1.16 ± 0.27*	1.12 ± 0.21*	1.05 ± 0.21*	1.41 ± 0.22*	1.18 ± 0.20*	1.19 ± 0.24*	1.07 ± 0.28*
12	1.86 ± 0.52	1.56 ± 0.54	0.79 ± 0.13*	1.45 ± 0.74	1.91 ± 0.54	1.66 ± 0.64	1.58 ± 0.73	1.41 ± 0.73
24	1.42 ± 0.33*	1.20 ± 0.31*	1.23 ± 0.30*	1.24 ± 0.36*	1.45 ± 0.38*	1.21 ± 0.35*	1.17 ± 0.35*	1.15 ± 0.42*
48	0.96 ± 0.20*	0.69 ± 0.14*	0.87 ± 0.11*	0.81 ± 0.28*	0.86 ± 0.17*	0.70 ± 0.16*	0.78 ± 0.18*	0.79 ± 0.19*

* Compared with the action time of 12 h, $P < 0.05$.

Table 4
Effect of different concentrations of hypoxia with the action time of 24 h on expression of RUNX2 and MDM21 protein.

Hypoxiaconcentration (ppm)	RUNX2 mRNA				MDM21 mRNA			
	Group A	Group B	Group C	Group D	Group A	Group B	Group C	Group D
0.000	1.47 ± 0.20*	1.39 ± 0.21*	1.37 ± 0.29*	1.35 ± 0.23*	1.59 ± 0.23*	1.47 ± 0.22*	1.45 ± 0.26*	1.40 ± 0.22*
0.001	1.99 ± 0.27*	1.83 ± 0.26*	1.79 ± 0.22*	1.75 ± 0.26*	1.88 ± 0.24*	1.98 ± 0.23*	1.89 ± 0.25*	1.68 ± 0.28*
0.010	4.17 ± 0.69	3.38 ± 0.65	2.57 ± 0.64	2.52 ± 0.74	3.76 ± 0.78	2.99 ± 0.74	2.88 ± 0.86	2.81 ± 0.55
0.060	2.31 ± 0.37*	1.89 ± 0.29*	1.86 ± 0.26*	1.66 ± 0.26*	2.30 ± 0.26*	2.00 ± 0.28*	2.04 ± 0.25*	2.05 ± 0.22*
0.100	1.65 ± 0.26*	1.34 ± 0.22*	1.63 ± 0.25*	1.71 ± 0.22*	1.69 ± 0.29*	1.58 ± 0.26*	1.78 ± 0.28*	1.59 ± 0.24*

* Compared with the concentration of 0.010, $P < 0.05$.

Table 5
Effect of 0.010 ppm hypoxia with different action time on expression of RUNX2 and MDM21 protein.

Hypoxiaconcentration (h)	RUNX2 mRNA				MDM21 mRNA			
	Group A	Group B	Group C	Group D	Group A	Group B	Group C	Group D
0	2.17 ± 0.28*	1.98 ± 0.25*	1.75 ± 0.22*	1.45 ± 0.22*	1.88 ± 0.18*	1.78 ± 0.13*	1.80 ± 0.16*	1.79 ± 0.32*
6	2.88 ± 0.31*	2.16 ± 0.21*	1.86 ± 0.28*	1.94 ± 0.21*	2.38 ± 0.24*	2.18 ± 0.20*	2.18 ± 0.24*	2.06 ± 0.31*
12	3.65 ± 0.66	2.45 ± 0.64	2.40 ± 0.65	2.15 ± 0.72	2.89 ± 0.67	2.69 ± 0.56	2.58 ± 0.64	2.41 ± 0.63
24	2.26 ± 0.29*	1.98 ± 0.24*	1.84 ± 0.24*	1.87 ± 0.22*	2.49 ± 0.34*	2.21 ± 0.24*	2.02 ± 0.25*	1.95 ± 0.38*
48	1.75 ± 0.28*	1.65 ± 0.28*	1.52 ± 0.21*	1.67 ± 0.24*	1.92 ± 0.32*	1.73 ± 0.26*	1.78 ± 0.28*	1.79 ± 0.39*

* Compared with the action time of 12 h, $P < 0.05$.

4. Discussion

The periodontitis is caused by the chronic infection of periodontal tissues of microorganisms and dental plaque, which can change the dentin and make the teeth loosen and fall. Many systemic diseases are closely related to the periodontitis. Meanwhile, the periodontitis could also cause the absorption of alveolar bone and gingivitis, destroy the periodontal tissue and then become the main reason of dental injury of healthy people [9–11]. The obstructive sleep apnea hypopnea syndrome is some kind of disease caused by many factors, with the main manifestation of sleep apnea. The decreased ventilation could cause a series of clinical response syndromes and its change was closely related to the endocrinology and metabolism and systemic diseases, as the independent risk factor of many cardiovascular diseases of cerebral stroke, myocardial infarction, hypertension and coronary heart disease [12,13]. In recent years, with the emphasis on the development of interdisciplines at home and abroad, the previous researches reported the close relationship between OSAHS and periodontitis. Especially, the occurrence of OSAHS in male patients with smoking and the age over 55 year-old was closely related to the periodontitis, which might further destroy the cementum of patients with periodontitis [4,14,15]. Therefore, in this study, it was to discuss the effect of chronic intermittent hypoxia on the expression of RUNX2 and MDM21 in rats with periodontitis, in order to understand the action of periodontitis in the destroy of dental bone tissues of rats in the condition of chronic intermittent hypoxia.

The transcription factor of runt domain gene family RUNX2 is also named as the core binding factor α1 and its expression is regulated by many growth factors and hormones that are involved in the osteoblastic differentiation. MDM21 is one of major inducing factors of osteoblastic differentiation and bone formation, which could activate the transforming growth factor receptor through the peroxisome proliferator and thus promote the osteoblastic differentiation [16]. According to previous researches, RUNX2 and MDM21 could promote the bone formation, mature of chondrocytes and production of osteocalcin, which could contribute to the osteoblastic differentiation jointly [17,18]. The results of this study indicated that, in the condition of hypoxia and according to the immunohistochemistry assay, the expression of RUNX2 and MDM21 was significantly decreased. The expression of RUNX2 and MDM21 was relatively high in the normoxic control group, but it was decreased in the normoxic periodontitis group, hypoxic control group and hypoxic periodontitis group in turn. According to the quantitative analysis of mean optical density, the mean optical density in the normoxic periodontitis group, hypoxic control group and hypoxic periodontitis group was significantly lower than that in the normoxic control group, with the statistical difference. But there was no statistical difference between the normoxic periodontitis group, hypoxic control group and hypoxic periodontitis group. The intermittent hypoxia condition of sleep apnea is the eased activation of different inflammatory reaction channel. As it can open the tissue injury and because of the periodontitis, the production and release of abundant cytokines and adherence factors can form the inflammatory cascade effect, which may aggravate the tissue injury and the abnormal expression of inflammatory factors. Afterwards, when the concentration was 0.010 ppm, the expression of RUNX2 and MDM21 in each group was higher than that in groups at other concentrations, with the statistical difference. Under the action with the concentration of 0.010 ppm for 12 h, the expression of RUNX2 and MDM21 was that normoxic control group > periodontitis group > chronic intermittent hypoxia group > compound group, but there was no significant difference for the comparison among groups ($P > 0.05$). With the increase in the concentration, the expression of RUNX2 and MDM21 would be decreased after reaching the peak, which might be related to the hypoxia concentration and action time. The increased concentration and prolonged action time would all affect the adequacy of metabolism inside and outside the cells and change the osmotic pressure. In case of great destroy by the metabolism,

the cell structure will be further destroyed, even the dissolution of cell membranes and programmed cell death. Thus in the clinical practice, it was not to grasp the concentration and time to reduce the side effects of long-term hypoxia, but it maintained the ossification of dentine and reduce the occurrence of dental bone loss caused by the periodontitis and OSAHS [5,19].

In conclusion, the expression of RUNX2 and MDM21 can be regarded as the reflection of injury degree of dentine of periodontal disease to the certain extent to provide the biological reference for the further study on the pathogenesis of dentine injury of patients with OSAHS and contribute to the prevention and treatment of patients with OSAHS and periodontitis during the injury of periodontal tissue. It is worthy to further discuss its specific mechanism.

Conflict of interest statement

We declare that we have no conflict of interest.

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