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Expression and distribution of TNF−α and PGE$_2$ of periodontal tissues in rat periodontitis model

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ABSTRACT

Objective: To simulate the expression of TNF−α and PGE$_2$ of periodontal tissues in rat periodontitis model. **Methods:** 40 Wistar rats were randomly divided into the periodontitis group and the control group (n=20). After the successful establishment of periodontitis rat model, raising for six weeks before the animals were sacrificed. The periodontal tissues were obtained and made into slices. Observed the histopathological changes of the periodontal tissues and measured TNF−α, PGE$_2$ levels change by immunohistochemistry, Western blot analysis and ELISA. **Results:** TNF−α, PGE$_2$ expression of the periodontitis group was significantly higher than that in the control group, the difference was significant (P<0.05). **Conclusions:** The TNF−α, PGE$_2$ expression of the rat periodontal tissue in the periodontitis group was significantly higher than the control group.

1. Introduction

Periodontal tissue is an important line of defense for the oral epithelial immune system[1−4]. Periodontitis is the most common infectious diseases which can affect the health of the periodontal tissue[5−7]. Periodontitis is caused by microorganisms such as G−anaerobes, which can affecte the periodontal tissue and activate of host defense cells to release a variety of inflammatory mediators, lead to secondary injury of periodontal tissue[8−10]. Therefore, inflammatory mediators play an important role in its occurrence and development, and the increasing number of studies suggest that periodontal disease is closely related to the level of inflammatory mediators[11−13]. However, the reports about the distribution and the level of inflammatory mediators of periodontal ligament and alveolar bone in patients with periodontiti are less. In this study, we established periodontitis rat model to observe the expression of TNF−α and PGE$_2$ in periodontal tissues and

analyzed, then determine the role of TNF−α and PGE$_2$ in the occurrence and development periodontitis.

2. Materials and methods

2.1. Materials

40 SPF male Wistar rats (200±20) g were purchased from Experimental Animal Center of XX University, TNF−α and PGE$_2$ ELISA kits were purchased from R & D Company. Rabbit anti−mouse TNF−α and PGE$_2$ antibody monoclonal antibody were purchased from Sigma (USA), fluorescent−labeled goat anti−rabbit secondary antibody was purchased from Thermo Company. TNF−α and PGE$_2$ immunohistochemistry kit was purchased from Beijing Bioss biotech company, total protein extraction kit was purchased from Invitrogen Corporation.

2.2. Methods

2.2.1. Grouping and modeling methods

40 male Wistar rats were randomly divided into the

periodontitis group and the control group, n=20. In the periodontitis group, rats were anesthetized with an intraperitoneal injection of 30 g/L of sodium pentobarbital (40 mg/kg), and the the limbs and head were fixed. Ligature wires were placed around the dental neck of upper first molar, and they were knotted at the lower jaw side of the gingival, tied up to avoid slipping, re–ligated if it fell off. Ligation wire was placed in gingival sulcus to avoid damage to the junctional epithelium. After anesthesia the rats were fed with high–sugar diet. Ampicillin were ground and put into drinking water for oral administration to suppress endogenous bacteria in rats, 20 mg daily for 3 d. In the control group, rats were anesthetized with pentobarbital, after feding with the same diet[14].

2.2.2. Periodontitis modeling

After 6 weeks of periodontal ligation, the mobility degree of maxillary first molar, the depth of periodontal pocket, the color, shape, quality changes of gingival, gingival index, bleeding index, with or without attachment loss, alveolar bone loss were observed. Criteria of periodontitis modeling were as follows: Gingival hyperplasia and red swelling, bleeding index 3–4, gingival index 2–3, periodontal pocket depth 1 mm or more. Light microscopy indicated significantly inflammation of the gingival tissue. There were a large number of lymphocytes and neutrophil infiltration of the lamina propria tissue which combined with the dental body, alveolar bone edges showed clearly osteoclasts and bone absorptive lacunae[15].

2.2.3. Immunohistochemistry methods

After rats were sacrificed, the maxillary tissue with the teeth and periodontal tissues were used as the samples. All samples were divided into two groups. After surgical resection, samples of one group were immediately placed in −80 ℃ low temperature refrigerator for Western–blotting. Another part were placed in 10% formaldehyde solution at 4 ℃ for 1 week after saline flush.They were washed with distilled water then placed in 10% EDTA for decalcification 40 d, rinsed in PBS buffer for 12 h, dehydrated, embeded and cut into 3–4 μ m sections for immunohistochemical examination of TNF–α , PGE$_2$ expression in periodontal tissues[16].

In negative control, PBS was used instead of primary antibody. Brown particles in the cytoplasm and/or nucleus were considered as TNF–α , PGE$_2$ protein positive staining, and stained color which was stronger than the background is positive, while no color or light brown background color in the cytoplasm were negative. Results were determined by the double–blind method, each slice was separately judged by two experienced pathologists, and analyzed by semi–quantitative method. Five typical slices were selected and observed under 400 $\times$ magnification. At least 1 000

cells were counted to obtain the mean value, results are calculated by the percentage of positive cells. Cells positive level ≥50% were considered as strongly positive (+++), 20%–49% as moderately positive (++), <20% as weak positive (+); Slice positive cells≥10% considered positive, <10% considered as negative (−)[17].

2.2.4. Western–blotting detection

Periodontal tissue was removed from the liquid nitrogen. 1 mL PBS was used to grind bone tissue on ice. The supernatant were abandoned after centrifugation, then was placed in lysis buffer and added with protein tissue extract. Total protein was extracted, and was incubated in rabbit anti–mouse TNF–α monoclonal antibody and PGE$_2$ monoclonal antibody for 2 h respectively, at 1:1 000 dilution. After the membrane was washed for three times, it was incubated in goat anti–rabbit secondary antibody for 1 h at room temperature, 1: 5 000 dilution, scanned by fluorescence system, using β –actin as internal reference. The grayscale integral value of each stripe was recorded by computer, statistical analysis was conducted by sample integral value/ internal reference integral value ratio[18].

2.2.5. ELISA detection method

TNF–α and PGE$_2$ was determined by ELISA kit, in accordance with the product instructions. Optical density value was measured, the system minimum accuracy was 1.0 ng/mL.

2.3. Statistical analysis

All data was analyzed with SPSS 17.0 software. t–test, χ^2 examination, probability methods and the Spearman rank correlation analysis were applied. P<0.05 was regarded as statistical significant difference.

3. Results

3.1. Periodontitis model observation

Periodontal indexes of the periodontitis group was significantly higher than the control group (P<0.05). Periodontitis group attachment epithelial cells were with varying degrees of erosion, periodontal ligament fibers degenerated, arranged in disorder or breakage, a large number of neutrophils and other inflammatory cell infiltration, large osteoclasts appear, proper alveolar bone was destroyed. In the control group, there was no pathological changes of the attachment epithelial cells and alveolar bone, periodontal ligament fibers and the cementoblast were neatly arranged, almost no neutrophils and other inflammatory cell infiltration (Table 1).

3.2. TNF– α , PGE$_2$ immunohistochemical staining results of periodontal tissues of rats in each group

Immunohistochemistry showed that TNF– α , PGE$_2$ expression of the periodontal tissues in the control group were negative. There were 12 positive TNF– α expression of periodontal tissues of 20 cases in the periodontitis group, the positive expression rate was 60.0%. There were 11 positive PGE$_2$ expression of periodontal tissues of 20 cases in the periodontitis group, the positive expression rate was 55.0%. Statistical analysis showed that periodontitis TNF– α and PGE$_2$ expression were significantly higher than that in the control group ($P<0.05$, Table 2).

3.3. Western blot detection of TNF– α , PGE$_2$ protein expression in rats periodontal tissues

Westem–blotting results showed that TNF– α protein relative expression of 20 cases in the periodontitis group (TNF– α / β –actin) was significantly higher than that) in the control group ($P<0.05$). The relative expression of PGE$_2$ protein (PGE$_2$/ β –actin) was significantly higher than the protein expression of the control group ($P<0.05$). (Table 3).

3.4. ELISA detection of TNF– α , PGE$_2$ release levels

The serum TNF– α , PGE$_2$ concentrations of the periodontitis group were significantly higher than those of the control group ($P<0.05$) (Table 4).

3.5. Correlation analysis of TNF– α and PGE$_2$ protein expression

The Spearman correlation analysis showed there was a positive correlation between the TNF– α protein expression and PGE$_2$ expression ($r=0.628$, $P<0.05$) (Table 5).

4. Discussion

Periodontitis is a common infectious oral disease which is a serious threat to oral health. Periodontitis may be

Table 1
Periodontal indexes in each group.

	Gingival index	Depth of periodontal pocket (mm)	Tooth mobility	Alveolar bone loss
Control group	0.61±0.06	0.21±0.02	0.60±0.06	0.81±0.07
Periodontitis group	2.22±0.20*	1.43±0.15*	1.32±0.15*	2.13±0.16*

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

Table 2
TNF– α , PGE$_2$ expression of rat periodontal tissue.

Groups	TNF– α positive					PGE$_2$ positive				
	–	+	++	+++	positive rate (%)	–	+	++	+++	positive rate (%)
Control group	20	0	0	0	0	20	0	0	0	0
Periodontitis group	8	3	5	4	60.0*	9	3	5	3	55.0*

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

Table 3
TNF– α , PGE$_2$ protein expression in rats periodontal tissues.

Groups	Number of cases	TNF– α	PGE$_2$
Control group	20	0.11±0.02	0.08±0.02
Periodontitis group	20	0.42±0.09*	0.54±0.12*

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

Table 4
Comparison of cytokine expression in serum levels of two groups.

Groups	Number of cases	TNF– α (ng/mL)	PGE$_2$ (ng/mL)
Control group	20	0.61±0.02	3.12±0.36
Periodontitis group	20	1.42±0.09*	23.54±2.73*

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

Table 5
Correlation between TNF– α protein expression and PGE$_2$ expression.

TNF– α	PGE$_2$	
	+	–
+	9	3
–	2	6

related to the infection of Gram–negative bacteria. Gram–negative bacterial can release endotoxin to re–infecting the host, usually starting from the edge of the gingival sulcus, and gradually infect to the deep part, even destroy periodontal ligament and alveolar bone. Therefore, in order to study the changes of periodontal tissue inflammation, the establishment of periodontitis animal models is an important method. In this experiment, Wistar rats were used as experimental animals. Because Wistar rats are often used as a model animal, and its molar region, alveolar ridge and periodontal tissue is similar to human[19–21].

This research method is use the silk ligation as the plaque fixator. The accumulation of a large number of bacterial can secrete toxins and enzymes to impact the periodontal barrier. After 6 weeks of periodontal ligation, there are varying degrees of erosion of the periodontal epithelial cells in the rat molar gingival, periodontal ligament fibers degenerated, periodontal ligament fibers degenerated, arranged in disorder or breakage, a large number of neutrophils and other inflammatory cell infiltration, large osteoclasts appear, proper alveolar bone was destroyed. According to the diagnostic criteria of periodontitis the rat model was successfully established[22–24]. TNF–α as a glycoprotein with a variety of biological activity, which is considered one of the most important medium of inflammation and immune response. Studies suggest that TNF–α is a cytokine produced by monocytes/macrophages which has a proinflammatory effect, the biological effects is only relevant with periodontal gum but also directly or indirectly mediated bone tissue absorption and inhibit bone formation[25].

The immunohistochemical result of this study showed that there is almost no TNF–α expression in the normal control group, the TNF–α positive expression of periodontal tissues of rats in the periodontitis group was significantly higher than that in the normal control group. Westem–blotting results also showed TNF–α protein expression of the periodontal tissues in the periodontitis group, ELISA results showed the expression of TNF–α in serum periodontitis group, the statistical analysis showed the difference had statistically significant. The result showed that if there is no inflammation, there is no activity expression, which remains in a stationary state. Its high expression can induce periodontitis in healthy oral environment, which is consistent with related findings. It is considered that the increased levels of serum TNF–α can promote inflammatory cells enter into the site of infection and induce the release of metalloproteinases, destroyed self–defense mechanism of periodontal tissue, eventually leading to the destruction of the periodontal collagen fibers and alveolar bone[26–28].

PGE$_2$ as a high level of inflammatory mediator can involve in the occurrence and development process of periodontal disease. Studies suggest that PGE$_2$ can significantly promote the proliferation and differentiation of osteoblasts, which not only involved in the proliferation and differentiation of osteoblast, but also regulate bone density and stimulate collagen synthesis. In this study, the immunohistochemistry, Westem–blotting and ELISA results all showed there is almost no expression of PGE$_2$ in the normal control group, while significantly increased in the periodontitis group, the difference is obvious. Therefore it is considered to be involved in the occurrence and development of periodontal disease in rats, previous literature showed that the mutual regulation of PGE$_2$ biosynthesis and TNF–α is often occurs in inflammation. The PGE$_2$ secretion increased during the process of alveolar bone loss which is induced by inflammation. PGE$_2$ increased secretion plays a crucial role to promote the expression of TNF–α activity[29].

This study showed that the expression of TNF–α and PGE$_2$ protein were positively correlated, which indicating that the biosynthesis of PGE$_2$ is close related to TNF–α activity. Maybe along with the periodontal disease, the number of bacteria around the peripheral edge will increase, then produce more TNF–α and PGE$_2$, thereby promoting the destruction of periodontal tissue and alveolar bone resorption, form a vicious circle aggravating, eventually deepened the periodontal pocket depth[30].

But the research is still in its early stages, only provide a theoretical basis for the pathogenesis and treatment of periodontal disease, setup a solid base for further research on the prevention and treatment of periodontitis.

In summary, we established the rat periodontitis model and the observated the PGE$_2$ and TNF–α expression of periodontal tissues. Preliminary, we think that periodontitis can release the inflammatory cytokines and promote the progress of periodontal tissue damage. However, the specific mechanism needs further study.

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] Hu XK, Peng H, Wang QZ, Chen WJ. Change of nerve growth factor in periodontal tissue during orthodontic tooth movement. *Oral Med* 2009; **29**(4): 205–207.

[2] Yao LY, Wang WY, Zhong Q, Yan FH. Experimental periodontitis model establishment in Sprague–Dawley rats and histological evaluation. *Fujian Med J* 2009, **31**(3): 63–65.

[3] Zhong LJ, Xu J, Zhang YM, Ni J, Zhou XH, Shi FC. Establishment and analysis of chronic periodontitis and atherosclerosis model in

Wistar rat. *Chin J Stomatol* 2009; **44**(8): 464–468.

[4] Qian YJ Zhong LJ, Mi CB, Li XB, Nie J, Pan X. The effect of orthodontic force on the inflammatory periodontal tissue remodeling and the expression of IL–6 in rats. *Chin J Stomatol Res* 2010; **4**(5): 447–455.

[5] Sun JF, Jing DB, Liu HJ, Li X, Wang LQ, Wang XP. Effect of *Lanthanum chloride* on experimental periodontitis in SD rats. *J Practical Stomatol* 2011; **27**(6): 743–748.

[6] Sun JJ, Wang D, Wang AQ. The establishment of the experimental periodontitis model in rats. *J Clin Rehabil Tissue Eng Res* 2012; **16**(37): 6867–6870.

[7] Trombone AP, Ferreira SB Jr, Raimundo FM. Experimental periodontitis in mice selected for maximal or minimal inflammatory reactions: increased inflammatory immune responsiveness drives increased alveolar bone loss without enhancing the control of periodontal infection. *J Periodontal Res* 2009; **44**(4): 443–451.

[8] O'Connell PA, Taba M, Nomizo A, Foss Freitas MC, Suaid FA, Uyemura SA, et al. Effeets of Periodontal therapy onglyeemie control and inflammatory markers. *Periodontol* 2008; **79**(5): 77–78.

[9] Knowles HJ, Athanasou NA. Acute hypoxia and osteoclast activity: A balance between enhanced resorption and increased apoptosis. *J Pathol* 2009; **218**(2): 256–264.

[10] Schierano G, Pejrone G, Brusco P. TNF–alpha TGF–beta2 and IL–1beta levels in gingival and peri–implant crevicular fluid before and after de novo plaque accumulation. *Clin Periodontol* 2008; **35**(6): 532–538.

[11] Zhao W, Yu ZH, Li XL, Yang Q, Gao CZ. Effects of ginsenoside Rg–1 on the expression of TNF–α and IL–1 β in periodontal tissues in rats. *Oral Med Res* 2009; **25**(5): 548–550,554.

[12] Zhang Y, Sun HT, Wang P. Observation of periodontitis model of rat bone resorption induced and natural outcome. *Chin J Conserv Dent* 2013; **23**(5): 294–297.

[13] Coimbra LS, Rossa C Jr, Guimarães MR, Gerlach RF, Muscará MN, Spolidorio DM, et al. In fluence of antiplatelet drugs in the pathogenesis of experimental periodontitis and periodontasl repair in rats. *J Periodontol* 2011; **82**: 767–777.

[14] Yu DH, Zhang GY, Zhang XH, Du JD, Yang Q, He FD. Effect of berberine hydrochloride on the expression of interleukin–1 β and tumor necrosis factor– periodontal tissues in rats. *Huaxi Oral Med J* 2008; **26**(1): 82–86.

[15] Geng WY, Tan YH, Shi GS. Severe periodontitis patients non irritant whole saliva IL–6 and TNF alpha test. *Chin J Conserv Dent* 2009; **10**(5): 527–531.

[16] Saxlin T, Suominen–Taipale L, Leiviska J, Jula A, Knuuttila M, Ylöstalo P. et al. Role of serum cytokines tumournecrosis factor–a and interleukin–6 in the association between bodyweight and periodontal infection. *Clin Periodontol* 2009; **36**(3): 100–105.

[17] Yang X, Xiang XR, Li CH, Huang XQ. Effect of tonifying Qi nourishing kidney mixture on peripheral levels of TNF– α

and IL–1 β and morphological changes of alveolar bone in periodontitis rats. *Third Military Med Univ J* 2011; **33**(13): 1378–1381.

[18] Gaibani P, Caroli F, Nucci C, Sambri V. Major surface protein complex of *Treponema denticola* induces the production of tumor necrosis factor alpha, interleukin–1 beta, interleukin–6 and matrix metalloproteinase 9 by primary human peripheral blood monocytes. *J Periodontal Res* 2010; **45**(3): 361–366.

[19] Lohr G, Beikler T, Podbielski A, Standar K, Redanz S, Hensel A. Polyphenols from *Myrothamnus flabellifolia* Welw. inhibit *in vitro* adhesion of *Porphyromonas gingivalis* and exert anti–inflammatory cytoprotective effects in KB cells. *J Clin Periodontol* 2011; **38**(5): 457–469.

[20] Holtfreter B, Schwahn C, Biffar R, Kocher T. Epidemiology of periodontal diseases in the Study of Health in Pomerania. *J Clin Periodontal* 2009; **36**(2): 114–123.

[21] Guimaraes MR, Coimbra LS, de Aquino SG, Spolidorio LC, Kirkwood KL, Rossa C, et al. Potent anti–inflammatory effects of systemically administered curcumin modulate periodontal disease *in vivo*. *J Periodontal Re*s 2011; **46**(2): 269–279.

[22] Goyal S, Jajoo S, Nagappa G, Rao G. Estimation of relationship between psychosocial stress and periodontal status using serum cortisol level: a clinico–biochemical study. *Indian J Dent Res* 2011; **22**(1): 6–9.

[23] Semenoff SA, Semenoff TA, Borges AH, Pedro FL, Sakai VT. Methodological model of chronic stress associated with ligature–induced periodontitis in rats: a radiographic study. *Braz Oral Res* 2010; **24**(4): 455–459.

[24] Xu L, Dong FS, Xu LH. To decide–defense element–1, 2 in experimental rats with gingivitis periodontitis model expressed in gingival epithelial tissue. *Chin J Conserv Dent* 2011; **21**(1): 6–10.

[25] Zhang GY, Yu ZH, Zhang XL, Du JD, Yang Q, He FD. Influence of TGF– β 1 on proliferation and differentiation of HPDLFs *in vitro*. *Shandong Med* 2008; **48**(21): 35–37.

[26] Zhang XH, Yu ZH, Zhang GY, Yang Q, Du JD, He DF. Effect of transforming growth factor– β 1 on the levels of il–6 and bgp in periodontal tissues in rats. *J Oral Sci Res* 2010; **26**(2): 171–174.

[27] Toygar HU, Kircelli BH. Regeneration of multiple adjacent bone dehiscences with guided tissue regeneration after orthodontic proclination: a corrective treatment approach. *Int J Periodontics & Restorative Dentistry* 2010; **30**(4): 345–353.

[28] Wang B, Liu D, Gong L. Clinical significance of cytokines level changes in gingival crevicular in patients with chronic periodontistis. *J Liaoning Med Univ* 2011; **32**(4): 308–313.

[29] Yang N, Wang CY. Tumor necrosis factor alpha's relationship with periodontitis. *J Chengde Med College* 2009; **26**(2): 198–201.

[30] Hu S, Zhang LJ, Bai HY, Tian YJ. Effect of electro–acupuncture at Zusanli point on tumor necrosis factor– α induced–multiple organ dysfunction in rats with sepsis. *Chin J Pathophysiol* 2010; **26**(2): 353–356.