Original Article

Study on the expression of IL-17, TIM-1 and TIM-3 and the mechanism of insulin resistance in the periodontal tissues of rats with periodontitis and obesity

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Abstract: To analyze the expression change and mechanism of insulin resistance of IL-17, TIM-1 and TIM-3 in the double rat model of obesity with periodontitis. According to the sample size of each group, the animal models of normal group (A group), periodontitis (B group), obesity (C group), obesity with periodontitis (D group) were established respectively. The periodontal tissues and pancreatic tissues were observed by HE staining; immunohistochemical (IHC) staining was used to determine the number of IL-17 positive cells and IRS-2 (insulin receptor substrate protein -2) and Caspase-3 positive cells; immunofluorescence double staining method was used to detect the expression of tryptase-positive MCs in TIM-1 and TIM-3. The expression level of IL-17 in the periodontal tissues of rats in D group was significantly higher than that of B group rats ($P<0.05$); and the level of IL-17 in the two groups showed a trend of increasing and then decreasing with the passage of time. There are both tryptase+MCs expression in TIM-1 and TIM-3 in gingival tissue with periodontitis, in D group, the double positive expression of tryptase-TIM-1 and tryptase-TIM-3 of mast cells in gingival tissue of rats was significantly higher than that of other groups ($P<0.01$). The expression of Caspase-3 in pancreatic islets of rats in group C was (0.216±0.015), compared with the D group (0.320±0.022), the expression level was significantly lower, the difference was statistically significant; however, the expression level of IRS-2 in the pancreatic islets of rats in group C was (2.210±0.011) decreased significantly ($P<0.05$). IL-17 can promote the development of periodontitis to some extent, and may be involved in the destruction and recovery of periodontal tissue; TIM-1 and TIM-3 play an important role in the pathogenesis and development of chronic periodontitis; and periodontitis leads to decrease of islet beta cells and decrease of function.

Keywords: Periodontitis, obesity, animal studies, inflammatory factors, pancreatic beta cells

Introduction

Obesity or overweight is an important cause of death in adults [1]; Periodontitis is one of the main causes of tooth loss in adults, and it has become a major disease affecting adult oral health [2]. Studies have shown that metabolic syndrome may lead to more severe and rapid destruction of periodontal tissue, and the inflammatory reaction caused by periodontitis will have a certain impact on metabolic diseases [3-5], therefore, in recent years, the relationship between periodontitis and obesity has become the focus of many scholars. There are studies that show [6, 7], the development of periodontitis is closely related to obese people with poor blood glucose control and the increase of body mass index. At the same time, the study also showed that [8, 9] the high expression of inflammatory factors plays an important role in the occurrence and development of systemic diseases. In this study, we investigate the correlation between insulin resistance and periodontitis in obese rats by studying the relationship among the expression change of IL-17, TIM-1 and TIM-3 and the pathological changes in different tissues of rats.

Materials and methods

Primary materials, reagents and instruments

Primary materials: 120 newly born male SD rats (Rats provided by Hubei University of
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Medicine) were randomly divided into 4 groups: normal group (A), periodontitis group (B), obesity group (C), obesity with periodontitis group, with 10 rats in each group.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Hubei University of Medicine.

Reagents and instruments

The reagents and instruments used in this study are shown in Table 1.

Methods

Establishment of animal model

The obesity rat model was established by injection of glutamate induced based on the previous study [10]. In brief, neonatal rats received subcutaneous injection of 3 mg MSG/g bodyweight at 1, 2, 3, 6, 7, and 8 d of age. After 12 weeks, the Lee’s index, blood pressure, fasting blood sugar level and oral glucose tolerance test (OGTT) were measured to determine the successful rat model of obesity, among which, fasting blood sugar level of 100 mg/dl to 125 mg/dl corresponds to a 2-hour glucose tolerance levels of 140 mg/dl to 199 mg/dl were considered as the indication of obesity. Periodontitis model was established by periodontal local ligation and coating periodontitis colonies. Ligation under the local anesthesia was carried out in the first and the second molar site in bilateral maxillary, and periodontal pathogen suspension was coated, modeling 8 w. The rats were delactated after birth 21 d with single cage feeding. During the construction of the animal model, the basic situation and daily activities of subjects were observed.

Specific operations are as follows: group A received no treatment; rats in group B and group D were treated by periodontal local ligation and coating periodontitis colonies; rats in group C and group D received subcutaneous injection of MSG.

Preparation of tissue samples

The rats were sacrificed with 10% chloral hydrate (1 ml/100 g) for death and fixation,

### Table 1. Reagents and instruments used in the study

<table>
<thead>
<tr>
<th>Reagent/Instrument</th>
<th>Manufacturer/Type/Concentration</th>
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<tbody>
<tr>
<td>Citrate buffer</td>
<td>0.01 mo/L, PH 6.0</td>
</tr>
<tr>
<td>Goat serum</td>
<td>Beijing Zhongshan Golden Bridge biotechnology Co., Ltd.</td>
</tr>
<tr>
<td>SP immunohistochemistry Kit</td>
<td>Beijing Zhongshan Golden Bridge biotechnology Co., Ltd.</td>
</tr>
<tr>
<td>DAB staining Kit</td>
<td>Beijing Zhongshan Golden Bridge biotechnology Co., Ltd.</td>
</tr>
<tr>
<td>Primary antibody dilution</td>
<td>Beijing Zhongshan Golden Bridge biotechnology Co., Ltd.</td>
</tr>
<tr>
<td>Antibody of mouse, Antibody of rabbit</td>
<td>Cell signaling technology, American</td>
</tr>
<tr>
<td>Rabbit anti mouse IL-17 polyclonal antibody</td>
<td>Hebei Bohai Biological Engineering Co.</td>
</tr>
<tr>
<td>Mouse Anti-Mast cell Tryptase</td>
<td>Abcam, Britain</td>
</tr>
<tr>
<td>Rabbit Anti-TIM-1</td>
<td>Wuhan boshide Biological Engineering Co. Ltd.</td>
</tr>
<tr>
<td>Rabbit Anti-TIM-1</td>
<td>Santa Cruz, American</td>
</tr>
<tr>
<td>Caspase-3 (1:200)</td>
<td>Santa Cruz, American</td>
</tr>
<tr>
<td>IRS-2</td>
<td>Abbiotec, American</td>
</tr>
<tr>
<td>Secondary Antibody solution</td>
<td>Biyuntian Biological Technology Research Institute, China</td>
</tr>
<tr>
<td>Poly-1-lysine slides off protection</td>
<td>Beijing World Tai Co., Ltd.</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Eppendorf, Germany</td>
</tr>
<tr>
<td>PH Tester</td>
<td>PHSJ-3F Shanghai KINGCO, China</td>
</tr>
<tr>
<td>Electronic balance, 0.0001 g</td>
<td>BP110S, sartorius, Germany</td>
</tr>
<tr>
<td>Magnetic thermostatic mixer</td>
<td>IKA, Germany</td>
</tr>
<tr>
<td>Constant temperature incubator</td>
<td>Heraeus, Germany</td>
</tr>
<tr>
<td>Ultra-low temperature freezer</td>
<td>Sanyo, Japan</td>
</tr>
<tr>
<td>Inverted microscope</td>
<td>Olypus, Japan</td>
</tr>
<tr>
<td>Low temperature centrifuge</td>
<td>Thermo, American</td>
</tr>
<tr>
<td>Ultra-pure water system</td>
<td>Mili-Q Ultra-Pure, Millipore, Billerica, MA, USA</td>
</tr>
</tbody>
</table>
periodontal tissue and pancreatic tissue were obtained. In brief, periodontal tissue at bilateral maxillary bones was collected and sectioned into 5 cm × 5 cm. 2-3 cm incision was made in the middle of rat abdomen. The pancreas located among stomach, liver, the small intestine, representing pale yellowish white tissue. The membrane between stomach and spleen was removed and then pancreatic tissue was collected.

Specific treatment on samples (volume around 1 mm³) is as follows: (1) The periodontal tissues were fixed with 4% paraformaldehyde in the treatment of 48 h with 0.9% Sodium Chloride Solution, decalcification was carried out on the condition of 10% EDTA solution for 4 weeks, then embedding and making continuous slices were prepared for future using. (2) The pancreatic tissue was removed and fixed with paraformaldehyde, the concentration and time were as follows for future using.

**Test method**

**HE staining**

Slice samples of periodontal tissue and pancreatic tissue of each group rats after dewaxing in the following steps to complete HE staining. (1) Anhydrous ethanol I, II, gradient alcohol solution of various concentration were used to soak for 5 min; (2) Hematoxylin staining was carried out for 5 minutes; (3) Differentiation was carried out by using 1% ethanol hydrochloride for 30 s; (4) Ammonia water was for 1 min; (5) Eosin staining was carried out for 10 min; (6) After gradual dehydration, transparent and mounting, observation was carried out. During the course of staining, it was not the first, seventh, eighth steps were completed until the use of water rushed to the slice without solution residue, after the rest of the steps are completed, PBS was used to rinse for 5 minutes, repeat for 3 times; at the same time, in the operation of the ninth step, should pay attention to the gradual dehydration.

**Immunofluorescence double staining**

Dewaxed sections were treated with toast in the condition of 62°C for 30 min, and then immunofluorescence double staining was completed through the following steps. (1) Sections were soaked in xylene solution I and II for 15 min; (2) The soaking time of slices in anhydrous ethanol I and II was 15 min and 5 min; (3) Slices were soaked for 5 min in the gradient of alcohol; (4) 3% H₂O₂ solution was used to treat for 20 min at a temperature of 37°C; (5) Under the condition of the temperature of 37°C, the serum was enclosed with a drop of liquid for treatment for 1 hours; (6) Under the condition of the temperature of 4°C, primary antibody was added (Mouse Anti-Mast cell Trypatase with 1:200 dilution + Rabbit Anti-TIM-1/Rabbit Anti-TIM-3 with 1:100 dilution), overnight; (7) Secondary antibody was incubated (Anti Mouse and Anti Rabbit both with 1:200 dilution) for 90 min; (8) Horseradish enzyme was labeled with streptavidin working solution for 15 min; (9) The slices were mounted and observed under the microscope. At the same time we should pay attention to the third step after the completion of the use of water wash section for 5 minutes, after completion the operation of fourth, sixth, seventh, and eighth steps, PBS solution was used to rinse for 5 min, which was repeated 3 times.

**Data statistics**

SPSS19.0 professional data processing software was used to analyze all the data, the mea-
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Results

Results of HE staining

Periodontal tissues: The collagen fibers were mainly distributed in group A, there was no obvious inflammatory cell infiltration and inflammatory exudates; there was an increase in the number of subepithelial fibrous connective tissue in group B, which showed focal lesion, a large number of chronic inflammatory cells such as monocytes, lymphocytes, plasma cell number also increased, which showed moderate inflammatory infiltration; the D group showed subepithelial collagen fiber degeneration, dissolution, and most of which has been replaced by inflammatory cells, with a sharp increase in the number of lymphocytes, as shown in Figure 1.

Pancreatic tissue: Islet were wrapped with thin layer connective tissue in group A, which had clear boundaries with exocrine glands; in group C: exocrine gland atrophied, fat cell deposited and replaced the exocrine glands; in group D: it was observed that boundary of the islet tissue was fuzzy, islet began to show fibrosis state, and some irregular fibrous tissue stretched into exocrine glands, as shown in Figure 2.

Immunohistochemical staining results

Expression of IL-17 in periodontal tissues: There was no significant difference between A and C groups at different time points. In B group, IL-17 was weakly positive in gingival and periodontal...
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membrane in group of the first day, but there was no significant difference between A and C group; there were stained osteoclasts and the expression of IL-17 increased in group of the third day; IL-17 had higher expression in both periodontal ligament and alveolar bone in group of the first week; a large number of osteoclasts appeared, with large number, strong staining, fiber necrosis and degeneration, IL-17 positive expression reached the peak in group of the second week; the osteoclasts of teeth reduced, staining osteoblasts were occasionally showed, IL-17 positive expression decreased, which was lower than that of group the fourth week. In D group, IL-17 was weakly positive in periodontal tissues on the first day. The positive expression of IL-17 was enhanced on the third day, which the expression intensity reached the highest point on the first week, and increased significantly. A large number of staining osteoclasts appeared, with destruction of bone resorption and formation of irregular severe defects, (Figure 3).

Expression of caspase-3 and IRS-2 protein in pancreatic tissue: Caspase-3 and IRS-2 proteins were labeled in each group of pancreatic islet, the expression of Caspase-3 (0.216±0.015) in group C was significantly lower than that the expression of pancreatic islets in D group (0.320±0.022); the expression of IRS-2 in pancreatic tissue of rats in group C was (0.299±0.010), compared with D group (2.210±0.011), which was significantly increased, P<0.05, specific data as shown in Table 2; Figures 4 and 5 are the results of HE staining of rat islets.

Table 2. Comparison of relative amount of Caspase-3 and IRS-2 protein in pancreatic islets

<table>
<thead>
<tr>
<th>Relative protein content</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
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<tbody>
<tr>
<td>Caspase-3</td>
<td>0.138±0.009</td>
<td>0.141±0.003</td>
<td>0.216±0.015</td>
<td>0.320±0.022</td>
</tr>
<tr>
<td>IRS-2</td>
<td>0.202±0.009</td>
<td>0.197±0.010</td>
<td>0.299±0.010</td>
<td>2.210±0.011</td>
</tr>
</tbody>
</table>

Figure 3. Specific changes of IL-17 in each group. Note: A: Staining results of pancreatic tissue in group A; B: Staining results of pancreatic tissue in group B; C: Staining results of pancreatic tissue in group C; D: Staining results of pancreatic tissue in group D.

Figure 4. Islet Caspase-3 markers in each group. Note: A: Caspase-3 labeling results in pancreatic islets tissues of group A; B: Caspase-3 labeling results in pancreatic islets tissues of group B; C: Caspase-3 labeling results in pancreatic islets tissues of group C; D: Caspase-3 labeling results in pancreatic islets tissues of group D.
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Results of immunofluorescence double staining

The density of double positive MCs of tryptase-TIM-1 and tryptase-TIM-3 in group B and group D were significantly higher than those in group A and C group (P<0.01); Tryptase-TIM-1 density of D group was significantly higher than that of A, B, C group (P<0.01), Figure 6.

Discussion

Periodontitis is a chronic progressive disease caused by pathogenic bacteria in the periodontal tissue, finally, it can lead to serious injury of periodontal supporting tissue, and cause inconvenience to the quality of daily life [11, 12]. When bacteria stimulate, periodontal tissue will secrete a large number of inflammatory factors to promote the occurrence of immune response [13]. It has been found that insulin resistance is closely related to the expression of some inflammatory factors in vivo [14, 15]; some studies have suggested that inflammatory factor such as interleukin also has a rising trend in obese people in vivo [16, 17]. Therefore, it has been suggested that obesity promotes inflammation or obesity itself is an inflammatory response [18]. In this study, we investigated the correlation between periodontitis and insulin resistance in obese rats by studying the relationship among the expression changes of IL-17, TIM-1 and TIM-3 and the related tissue lesions in different tissue models of rats.

The results of HE staining showed that there was no obvious inflammatory cell infiltration and inflammatory exudate in group A; in group B, there was an increase in the number of subepithelial fibrous connective tissue, showing focal shape, large number of chronic inflammatory cells were found in the bundle, and the density of plasma cells could be observed; which showed moderate inflammatory infiltration; in group D, epithelial collagen degenera-
tion and lysis were observed, some of them have been replaced by inflammatory cells, a large range of infiltration appeared, and inflammation was further aggravated; islets of group A was wrapped with thin layer of connective tissue, which had clear boundaries with exocrine glands; exocrine gland of group C atrophy, deposition of fat cells replaced the exocrine gland cells; by HE staining, it was also observed that the islet boundary of group D was blurred, and there was fibrosis, it can be seen that the boundary of the pancreatic islets is irregular and the exocrine glands extend; islet fibrosis appeared; this suggests that obesity may promote the development of periodontitis and periodontitis may also contribute to the damage of islet cells.

IL-17 factor can be secreted by many kinds of cells in periodontal tissue, but its main site of expression is Th17 cells, which were widely distributed in periodontal tissues of patients with periodontitis, research confirmed [19, 20] that IL-17 plays an important role in the development of many chronic inflammatory diseases, it is a kind of proinflammatory factors. Recent study identified the pathogenic effect of IL-17A on obesity-related inflammatory diseases [21]. Research also indicated that MAIT cells displayed an IL-17(+) phenotype in both obese adults and children, correlating with levels of insulin resistance [22]. In the process of periodontitis, IL-17 can promote and induce the expression of a variety of other cytokines on the basis of its own role, thus speeding up the process of inflammatory reaction in periodontitis, the periodontal tissues were destroyed [23]; in addition, IL-17 can also interact with IL-1β and tumor necrosis factor (TNF), thus, inflammatory factors produced synergy. IL-1β and TNF have long been shown to play an important role in insulin resistance in obese rats, which showed positive correlation between them [24]. The results of this study showed that In addition to the group A and the group C, in group B and group D, the changes of IL-17 at different time points increased first and then decreased. In this study, the IL-17 in periodontitis group was weakly positive in the periodontal tissues of the early stage of the disease, however, with the positive expression of IL-17 increased, osteoclasts appeared, and the inflammatory reaction increased, on the fourth week, HE expression of IL-17 reached peak, and the inflammatory response reached peak. In the early stage of group B, the expression of IL-17 was weakly positive, the gingival connective tissue appeared neutrophil infiltration; from the third day, there was significant difference between B group and group A, group C, inflammation increased, the expression of IL-17 increased, osteoclast cells appeared; on the first week, the expression of IL-17 reached peak, the range expanded to the periodontal ligament and alveolar bone, the inflammation was further aggravated, the neutrophils and osteoclasts increased; the positive expression of IL-17 reached the peak on the second weeks, and a large number of osteoclasts accumulated in periodontal tissues with strong staining, inflammation and tissue destruction; the positive expression of IL-17 decreased gradually, and the inflammation decreased, neutrophil reduced, tissue was repaired, osteoclast reduced. The development of periodontitis in group D was similar to that in group B; and compared with concurrent experimental group, on the third day, first week, second week, fourth week periodontitis group was significantly difference, the results suggest that the expression of IL-17 increases with the severity of periodontitis, and it also suggests that obesity may play a positive role in the development of periodontitis. The main role of TIM-1 is the synergistic effect of stimulating molecules on the immune response, at the same time, TIM-1 influences the release of cytokines, such as regulating macrophage cytokine production [25]. It has been demonstrated that TIM-1 and TIM-3 promoted the Th2 cytokine production through the effects of mast cells [26]. TIM-3 can be expressed on the surface of a variety of immune cells and participate in the regulation of immune function of these immune cells. Dysregulation of Tim-3 expression on innate immune cells leads to an excessive or inhibited inflammatory response and subsequent autoimmune damage or viral or tumor evasion [27]. The results of immunofluorescence double staining showed that tryptase-TIM-1 and tryptase-TIM-3 double positive MCs density in group B and group D were significantly higher than those in A and C two groups; the expression MCs density of tryptase-TIM-1 and tryptase-TIM-3 in the periodontal tissues of rats in group D was significantly higher than that in group B. This suggested that TIM-1, TIM-3 periodontitis gingival
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tissue expression of tryptase+MCs, and with the increase in the severity of periodontitis and increased expression density.

Poly ribose polymerase is an important material related to DNA repair and gene integrity, it is also an important substrate for Caspase-3, therefore, when the expression of Caspase-3 is increased, the consumption of poly polymerase will be aggravated, so that the apoptosis of cells will increase; While IRS-2 is mainly distributed in the liver and pancreatic β cells, its main function is to promote the effect of liver glycogen synthesis and inhibiting glycogen output, therefore, the expression of IRS-2 was decreased in the obese rats with periodontitis, suggesting that periodontitis and obesity may promote the development of each other, but the specific mechanism still needs to be tested.

In conclusion, the expression of IL-17, TIM-1 and TIM-3 in gingival tissue of rats with periodontitis and obesity were significantly higher than those in normal rats, periodontitis and obese rats, moreover, the expression level of IL-17 in periodontal tissue of periodontitis and periodontitis combined with obesity showed a trend of increasing and then decreasing with the passage of time. The expression of Caspase-3 was increased and the expression of IRS-2 was decreased in the obese rats with periodontitis, suggesting that periodontitis and obesity may promote the development of each other, but the specific mechanism still needs to be tested.

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Disclosure of conflict of interest

None.

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