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EXPERIMENTAL STUDY

Periodontitis aggravates kidney damage in obese mice by MMP2 regulation

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ABSTRACT

OBJECTIVE: To investigate the effect of periodontitis on the development of kidney damage in obese mice and its possible mechanism.

METHODS: C57 BL/6J mice were fed high-fat (HF) or low-fat (LF) diet and then divided into four groups: obesity with periodontitis (HFP), obesity without periodontitis (HFC), normal mice with periodontitis (LFP) and normal mice without periodontitis (LFC). Serum indicators of renal function, namely serum total protein (TP), albumin (ALB), creatinine (Cr) and blood urea nitrogen (UREA) were measured. The histopathological examination of kidney tissues was performed. The expressions of transforming growth factor-β1 (TGF-β1), matrix metalloproteinase-2 (MMP2) and tissue inhibitors of metalloproteinases-1 (TIMP1) were detected by immunohistochemistry and real time RT-PCR.

RESULTS: Obesity decreased TP and ALB, and increased serum Cr and UREA levels in normal and periodontitis mice groups, as well as induced glomerular and tubulointerstitial pathologic changes. Tubulointerstitial fibrosis was more severe in HFP group. In obese mice, periodontitis caused the downregulation of MMP2, and upregulation of TIMP1 and TGF-β1 at transcriptional and translational levels.

CONCLUSIONS: In obese mice, periodontitis may aggravate pathological changes in the kidney. The possible mechanism might lie in downregulation of MMP2 and upregulation of MMP inhibitor, TIMP1, and TGF-β1 *(Tab. 1, Fig. 4, Ref. 16)*. Text in PDF *www.elis.sk*.

KEY WORDS: obesity, periodontitis, kidney disease, MMP2.

Introduction

The morbidity rate of periodontitis, a common chronic disease, is about 47.6 % in USA, and higher in China. The chronic periodontitis is a nonspecific chronic inflammation caused by a biofilm, which is characterized by destruction of periodontal tissue, absorption of alveolar bone and deepening of periodontal pocket. Periodontitis has been recognized as a significant and common cause of low-grade chronic inflammation. This inflammatory state is not only localized, but also affects the whole body through complex inflammatory responses. The interaction between periodontal and systemic diseases has been the focus of numerous studies, including chronic kidney disease (CKD) which is a major public health burden in the USA and many other countries (1, 2). Increasing epidemiological evidence supports an association between periodontal disease and CKD (3–7). According to a clinical research of our team, 18.2 % of Chinese patients with the periodontal disease have proteinuria, hematuria, or reduced estimated glomerular filtration rate (eGFR), indicating the presence of kidney damage (8). The study of structural equation modeling supports a bidirectional relationship between CKD and periodontal disease (9). The increasing prevalence of obesity over the past decades is another important health problem (10), which enhances the risk of several diseases including diabetes, hypertension, cardiovascular disease, metabolic syndrome, certain malignancies, and CKD (11). Obesity participates in the genesis of CKD by predisposing to diabetic nephropathy, hypertensive nephrosclerosis, and focal and segmental glomerular sclerosis (12).

The evidence above has suggested that periodontitis and obesity is separately related to kidney disease. However, few studies deal with the effect of periodontitis on the kidney in obese animal models. In this study, we have induced the periodontal disease by periodontal ligation in a diet-induced obesity (DIO) mouse model, an animal model widely used for the study of obesity. Our goal is to explore the possible effects and mechanism of periodontitis in obese mice on kidney disease by using a periodontitis-and-obesity combined mouse model. By discussing the possible interaction between periodontitis and chronic kidney disease in obesity, we have tried to establish reliable evidence for clinical multidisciplinary treatment of periodontitis.

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Methods

Ethics statement

Animals were provided by and cared at the Guangdong Medical Laboratory Animal Center. The study was approved by the Ani-

Tab. 1 Serum indicators of the renal function.

Serum indicators	HFP	HFC	LFP	LFC	F	p
TP (g/L)	48.2±7.2	53.4±2.0	60.8±2.7	61.2±2.7	10.7	0.000
ALB (g/L)	31.0±6.7	34.7±5.2	44.4±2.6	47.8±6.5	10.4	0.000
Cr (μmol/L)	12.0±1.7	10.7±1.2	8.8±1.9	9.2±1.3	4.9	0.012
UREA (mmol/L)	11.3±2.5	10.7±2.7	8.7±1.1	7.8±2.5	2.5	0.092

mal Experimental Committee of Southern Medical University and in compliance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Animals

C57 BL/6J mice, 6 weeks old, male, were randomly divided into two groups: high-fat (HF) and low-fat (LF) diet groups. HF group was fed by high-fat diet for 30 weeks (30w) to induce obesity (DIO), while LF group was fed by low-fat diet as the control. At the end of each time point, the mice in each diet group were divided into periodontitis group (P) and non-periodontitis group (C). Periodontitis was induced by placing silk ligatures pre-soaked in Pg-growing broth for 24 h around both second maxillary molars, while non-periodontitis group was sham-ligated at the same sites with a sterile silk which was removed at once. The induction time was 5 or 10 days. Numbers of mice in each group were as follows: 30w 10d: n=18 in HFP, n=18 in HFC, n=12 in LFP, n=10 in LFC; 30w 5d: n=18 in HFP, n=18 in HFC, n=11 in LFP, n=10 in LFC. All mice were sacrificed by cervical dislocation. The right and left kidneys were removed, dissected, freed of adipose tissues, and weighed. A half of both the left and right kidneys were snap-frozen in liquid nitrogen. The other half of kidney was fixed in 4% paraformaldehyde for later histopathological examination.

Serum indicators of renal function

Before the mice were sacrificed, the blood samples had been collected by extracting eyeballs. Serum total protein (TP), albumin (ALB), creatinine (Cr) or blood urea nitrogen (UREA) was measured by a third-party medical laboratory company (Kingmed Diagnostics, Guangzhou, China). TP and ALB levels were separately measured by the biuret and BCG methods. Cr and UREA levels were measured by the enzymatic method.

Histopathological examination

For light microscopy examination, fixed kidney samples were embedded in paraffin wax. Sections (4 μm) were cut and stained with Masson.

Immunohistochemistry (IHC)

Immunohistochemistry was performed with two-step methods. Sections were incubated with primary antibodies against mouse TGF-β1 (1 : 400), MMP2 (1 : 3200), and TIMP1 (1 : 400). For blank control, primary antibodies were replaced with phosphate buffered saline (PBS). For color reaction, 3,3'-diaminobenzidine (DAB) (DAKO ChemMate EnVision) was used. Immuno-stained sections were counterstained with hematoxylin. The slides were observed with microscope (Olympus CX41), and the optical density value was measured by Image-Pro Express version 6.0 software

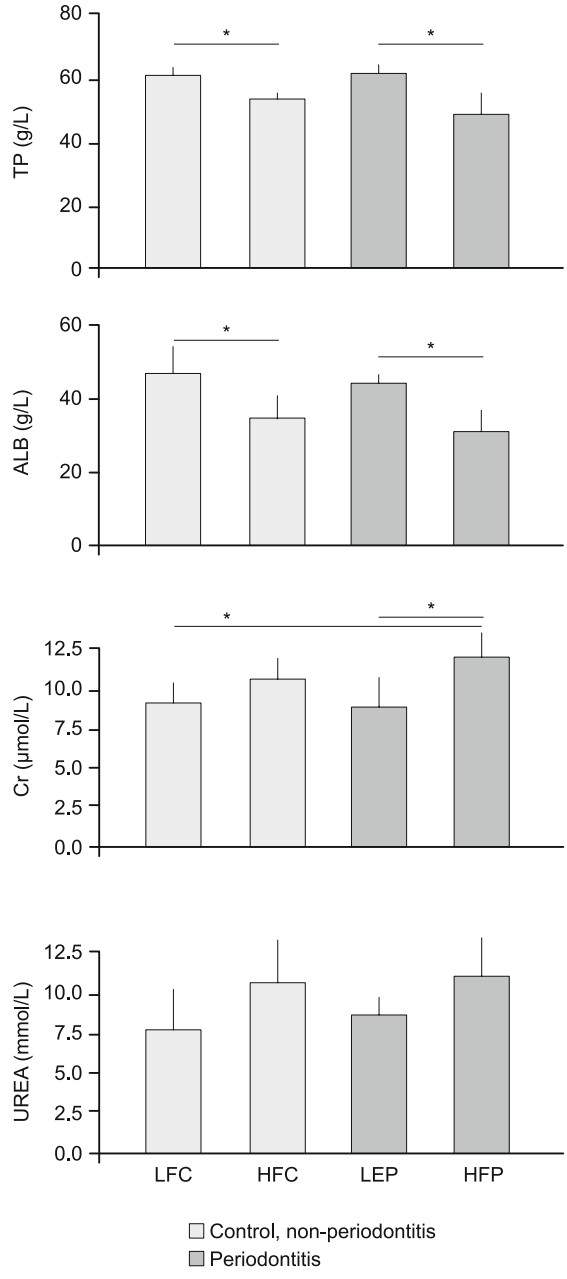

Fig. 1. Effects of obesity and periodontitis on the levels of serum proteins. Before the mice were sacrificed by cervical dislocation, the blood samples had been collected by extracting eyeballs. The concentrations of serum total protein (TP) (g/L), albumin (ALB) (g/L), creatinine (Cr) (μmol/L) and blood urea nitrogen (UREA) (mmol/L) were measured. ANOVA test, * $p < 0.05$.

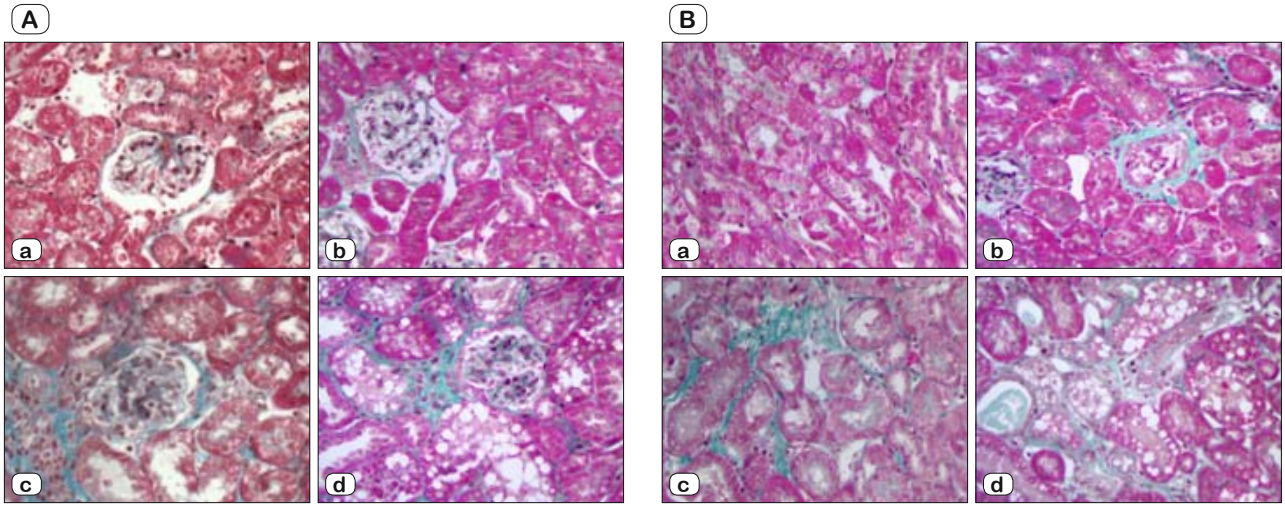

Fig. 2. Effects of obesity and periodontitis on renal histopathological changes. Sections (4 μm) of kidneys in 4 different groups were stained with Masson for light microscopy examination (Magnification 400x). A – Glomerulus; B – Renal tubule; Groups: LFC (a), LFP (b), HFC (c) or HFP (d).

(Olympus Sales & Service Co., Ltd., Guangzhou Branch), using the parameter of integrated optical density (IOD)/AREA, while the average and variance were calculated.

RNA isolation and RT-PCR

Expression of TGF-β1, MMP2, TIMP1 mRNA were detected by real-time reverse transcription (RT)-PCR. Total cellular RNA was extracted from mouse renal tissues and reversely transcribed (M-MLV Reverse Transcriptase, Invitrogen). Primer sequences for TGF-β1, MMP2, and TIMP1 genes were as follows: TGF-β1: forward 5'-CAT GGA GCT GGT GAA ACG GA-3'; reverse 5'-GGC GAG CCT TAG TTT GGA CA-3'; MMP2: forward 5'-TTT CTA TGG CTG CCC CAA GG-3', reverse 5'-GTC AAG GTC ACC TGT CTG GG-3'; TIMP1: forward 5'-ACG AGA CCA CCT TAT ACC AG-3', reverse 5'-GCT TTC CAT GAC TGG GGT GT-3'. GAPDH was used as the control. The reversely transcribed product was quantitatively determined with Faststart Universal SYBR Green Master (Rox) (Roche). The expression levels were calculated using the comparative Ct method.

Data analysis

The statistical analysis was performed using the statistical software SPSS 20.0. Data were presented as mean±SE. Normality was confirmed after checking all variable through normality test. Analyses were performed with ANOVA of factorial design. If there was an interaction effect, one-way ANOVA was used to analyze the simple effect of each factor. The p value of 0.05 was considered significant.

Results

The high-fat diet but not periodontitis affects the levels of serum proteins

To investigate the possible interaction between periodontitis and chronic kidney disease in obesity, we induced periodontitis in mice of the high-fat diet-induced obesity (DIO) and control (low-fat diet) groups as reported in the previous studies (13, 14). We observed whether the high-fat diet or periodontitis affected the levels of serum proteins as serum indicators of renal function (Table 1), and then we performed Student-Newman-Keuls (SNK) method for multiple comparisons. In non-periodontitis (C) or periodontitis (P) groups, the levels of serum total protein (TP) or albumin (ALB) in high-fat groups (HFP and HFC) decreased more significantly than that in low-fat groups (LFP and LFC) ($p < 0.01$). However, levels of TP and ALB were not significantly different between HFP and HFC ($p > 0.05$), or between LFP and LFC ($p > 0.05$) (Tab. 1 and Fig. 1 A, B). Serum creatinine (Cr) in HFP was significantly higher than that in LFP and LFC ($p < 0.05$), but there was no significant difference among HFC, LFP and LFC ($p > 0.05$), or between HFC and HFP ($p > 0.05$) (Tab. 1 and Fig. 1C). The levels of blood urea nitrogen (UREA) in HF groups were increased, but there was no significant difference among the four groups ($p > 0.05$) (Fig. 1D). These data suggest that, firstly, the high-fat diet significantly decreases the levels of serum TP and ALB, but increases the levels of Cr and UREA, and secondly, periodontitis affected the levels of serum proteins neither in the control (non-periodontitis) nor periodontitis groups.

The high-fat diet but not periodontitis is the main factor of renal pathological changes

The histological features in mice of LFP (Fig. 2Ab, Bb) were similar to LFC (Fig. 2Aa, Ba) while showing mainly normal renal histological features. In HFC (Fig. 2Ac) and HFP (Fig. 2Ad) groups, there was hyperplasia in glomerular mesangial cells and matrix, and the glomerular capillary loop partly disappeared. In HFC (Fig. 2Bc) and HFP (Fig. 2Bd) groups, there were also tubulointerstitial fibroplasia, vacuolar degeneration in cortical tubular epithelial cells and protein cast. The pathologic changes in HFC and HFP were almost similar, but tubulointerstitial fibroplasia was more severe in HFP group (comparing between Fig. 2Bc and d). By comparing groups of 5 d and 10 d periodontitis, we found that tubulointerstitial fibroplasia in HFP 10 d was more severe than that in HFP 5 d.

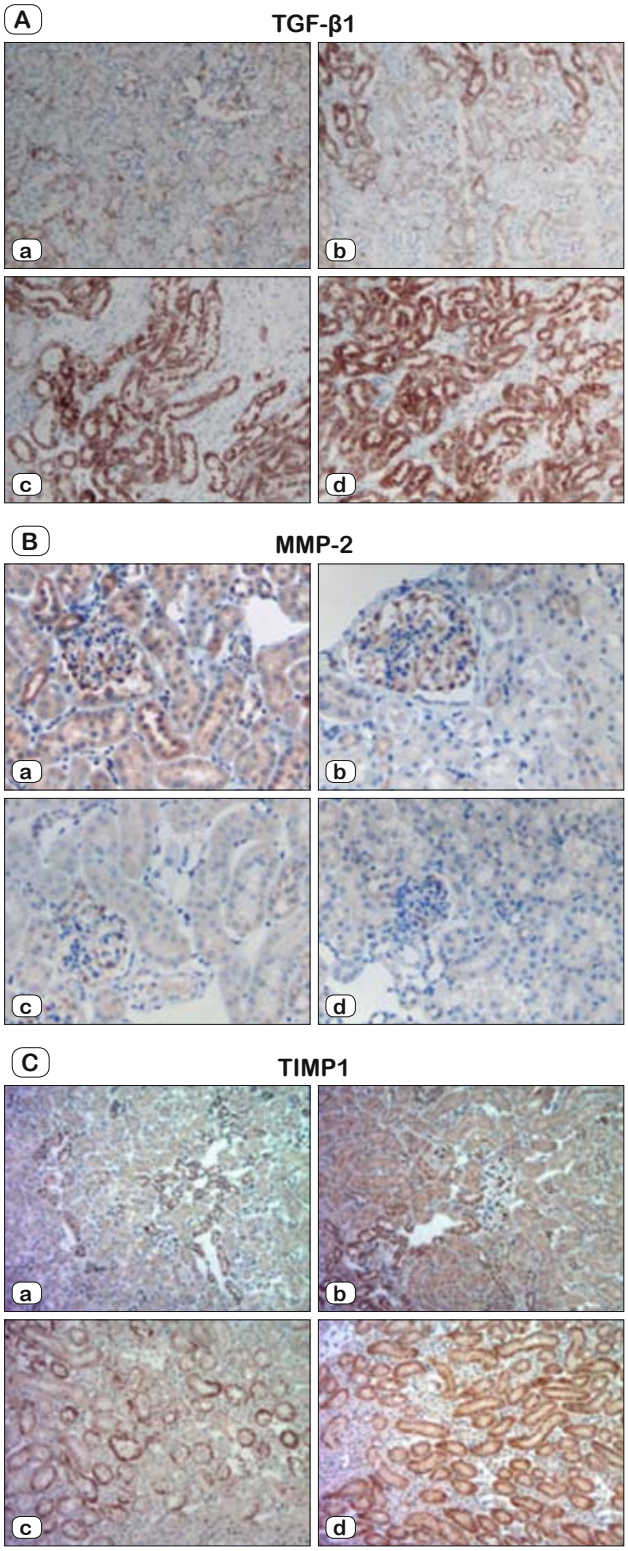

Fig. 3 . Effects of obesity and periodontitis on the expression of TGF-β1, MMP2 and TIMP1 in the kidney. Sections (4 μm) of kidneys in 4 different groups were stained with antibody against TGF-β1 (A), MMP2 (B) or TIMP1(C) and then secondary antibody HRP-labeled for light microscopy examination (Magnification 400x for MMP2, Magnification 200x for TGF-β1 and TIMP1). Groups: LFC (a), LFP (b), HFC (c) or HFP (d).

Even renal tubular structural destructions could be observed in HFP 10 d; hyperplasia in glomerular mesangial cells and matrix, and vacuolar degeneration in cortical tubular epithelial cells were not obviously different between HFP 10 d and HFP 5 d. While LFP 10 d and LFP 5 d showed mainly normal renal histological features, no significant difference was observed. The results of histological analysis reveal that periodontitis did not cause kidney lesion in low-fat diet mice, while obesity was the main factor of renal changes.

Periodontitis affects the expression of TGF-β1 but the high-fat diet affects the expression of TGF-β1, MMP2 and TIMP1

To investigate the possible mechanism of periodontitis and/or obesity causing the renal changes, we performed immunohistochemistry to analyze the expression of TGF-β1, MMP2 and TIMP1 in the kidney. Integrated optical density (IOD)/AREA was measured for semi-quantitative analysis and ANOVA was performed for statistical analysis. TGF-β1 is a marker of renal matrix accumulation, expressed in proximal convoluted and distal convoluted tubules. The expression of TGF-β1 is weak in renal glomerulus. Immunohistochemistry showed that there was a significant difference among groups HFP, HFC, LFP and LFC ($F=301.8$, $p < 0.01$). According to multiple comparisons by SNK method, the expression of TGF-β1 was significantly different between each two groups in a descending order as follows: HFP>HFC>LFP>LFC. The expression of TGF-β1 was not significantly different between HFP 10 d and 5 d ($p > 0.05$) (Fig. 3A).

MMP-2 is expressed in the renal mesangial and endothelial cells and proximal tubules. Since there was an interaction between two influencing factors (obesity and periodontitis time) for MMP2, the simple effect of each factor was further analyzed using one-way ANOVA. For the simple effect of obesity there was a significant difference among four groups HFP, HFC, LFP, and LFC, both in periodontitis 10 d ($F=123.6$; $p < 0.01$) and 5 d ($F=333.9$; $p < 0.01$). The highest expression was found in the LFC group. In HFP group, MMP-2 positive staining area was not obvious. According to multiple comparisons by SNK, the expression of MMP2 was significantly different between HF groups and LF groups, but no significant difference was found between HFP and HFC, indicating that periodontitis may not decline the MMP2 expression in the kidney of obese mice. Therefore, there was no need to further analyze the simple effect of periodontitis time (difference between 5d and 10d periodontitis) (Fig. 3B).

The expression of TIMP1 was found in the proximal tubules, distal convoluted tubules, and renal interstitial and glomerular matrix regions. There was a significant difference among HFP, HFC, LFP and LFC groups ($F=240.5$; $p < 0.01$). Multiple comparisons by SNK revealed significant differences between each two groups in a descending order as follows: HFP>HFC>LFP>LFC. The expression of TIMP1 was not significantly different between HFP 10 d and 5 d ($p > 0.05$) (Fig. 3C).

Periodontitis increases TGF-β1 and TIMP1 and decreases MMP2 mRNA expression

We further examined and compared the expression levels of TGF-β1, MMP2 and TIMP1 by real-time RT-PCR. By 30 w

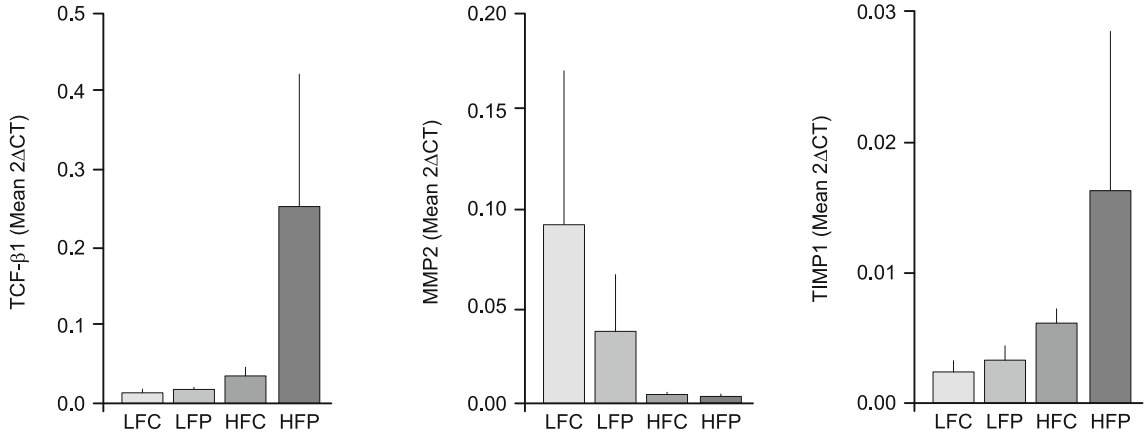

Fig. 4. Effects of obesity and periodontitis on the expression of TGF-β1, MMP2 and TIMP1 at the transcriptional level. Real time RT-PCR was performed to calculate the target gene expression in the test sample=$2^{-\Delta\Delta CT}$. The mRNA level of each group was normalized to the level of LFC, and presented as a fold increase (TGF-β1 and TIMP1) or percentage decrease (MMP2). Post hoc ANOVA test, * p < 0.05, ** p <0.01.

10 d treatment, TGF-β1 was expressed 1.00±0.29, 1.28±0.36, 2.25±0.87 and 16.38±8.08-fold in LFC, LFP, HFC, and HFP groups, respectively, suggesting that high-fat diet and periodontitis were significantly increased in TGF-β1 mRNA level (Post hoc ANOVA, $p < 0.01$) (Fig. 4A). MMP2 was expressed in 100.00±60.98 %, 41.91±32.15 %, 7.21±6.43 % and 3.54±2.66 % in LFC, LFP, HFC and HFP groups, respectively, suggesting that high-fat diet and/or periodontitis were significantly decreased at MMP2 mRNA level (Post hoc ANOVA, $p < 0.01$) (Fig. 4B). TMP1 was expressed 1.00±0.68, 1.39±0.57, 2.36±1.17 and 6.10±3.11-fold in LFC, LFP, HFC and HFP groups, respectively, suggesting that high-fat diet and periodontitis were significantly increased at TMP1 mRNA level (post hoc ANOVA, $p < 0.01$) (Fig. 4C). We further performed the factorial analysis. For TGF-β1 and TIMP1 results, there was no interaction among two influencing factors, namely obesity and periodontitis time (TGF-β1: $F=0.114$, $p=0.952$; TIMP1: $F=0.078$, $p=0.972$). Therefore, only the main effect was analyzed. The main effect of periodontitis time was not significant (TGF-β1: $F=0.338$, $p=0.562$; TIMP1: $F=0.833$, $p=0.363$), but there was a significant difference among HFP, HFC, LFP and LFC groups (TGF-β1: $F=62.652$, $p=0.000$; TIMP1: $F=37.472$, $p=0.000$). The mRNA expression level descended as follows: HFP>HFC>LFP>LFC, which corresponds to the immunohistochemistry results. The fold change between HFP and HFC groups was as follows: TGF-β1=7.293; TIMP1=2.587. The results indicate that periodontitis may upregulate the expression of TGF-β1 and TIMP1 in obese mice. There was an interaction between two influencing factors (obesity, periodontitis time) for MMP2 ($F=2.719$, $p=0.047$). For the simple effect of obesity, there was a significant difference among four groups (HFP, HFC, LFP and LFC) both in 10d ($F=18.465$, $p=0.000$) and 5d ($F=19.560$, $p=0.000$) periodontitis time. The highest expression of MMP2 was found in the LFC group, which corresponds to the immunohistochemistry results. The fold change in expression between the HFP and HFC group is 0.478. The results indicate that periodontitis may downregulate the expression of MMP2 in obese mice. For the simple effect of periodontitis time, in HFP group, the expression of MMP2 was not significantly different between HFP 10d and 5d ($p=0.069$).

Discussion

In the present study, our hypothesis was that periodontitis induced the kidney disease in obese animals. Reportedly, obesity can endanger CKD in several ways, including by development of diabetic nephropathy and hypertensive nephrosclerosis. In addition, obesity is associated with an increased risk of developing focal and segmental forms of glomerulosclerosis (16). Chronic periodontitis is caused by biofilm in the periodontal support tissue, and periodontal pockets filled with a large number of bacteria and inflammatory exudate. Recent studies revealed that both obesity and periodontitis are characterized by a low-grade chronic inflammatory state. In order to test our hypothesis, we have induced periodontitis in the diet-induced obesity (DIO) mouse model, a typical animal model for studying obesity (15).

We have found that periodontitis does not cause significant changes in function and histopathology in the normal mouse kidney, but promotes kidney glomerular mesangial cells and matrix hyperplasia, tubulointerstitial fibroplasia and renal interstitial fibrosis in obese mice, thus aggravating kidney disease and deteriorating the kidney function. In periodontitis-and-obesity combined mice, serum, TP and ALB decreased while Cr and UREA increased. In the presence of obesity, not only the periodontitis promotes the fibrogenic core factor TGF-β1 expression in the kidney tissue, it also increases the serum TGF-β1 level. In the obesity mouse model, periodontitis also contributes to high expression of TIMP-1 which is an inhibition factor of collagen degradation enzymes, including MMP2, leading to an increase in type IV collagen deposition in the kidney tissue. Our findings suggest that periodontitis may play a role in breaking the metabolic balance of extracellular matrix in the kidney by increasing collagen production and decreasing collagen degradation in obesity, thus resulting in the impairment of the kidney function.

In conclusion, our study provides the evidence that periodontitis may aggravate the pathologic changes in the kidney in an obesity mouse model. The possible mechanism lies in up-regulation of TGF-β1 and TIMP1, as well as down-regulation of MMP2. Therefore, the treatment of periodontitis may be beneficial in preventing chronic kidney disease.

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