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Original Article

Periodontitis regulates renal impairment in obese mice via TGF-β/Smad pathway

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Abstract: Objective: To determine the impact of periodontitis on renal impairment induced by obesity. Methods: Periodontitis and obesity models were induced using silk ligatures with bacteria and high-fat diet, respectively. Indicators of renal function were compared. Renal tubular epithelial cells (RTECs) were treated with lipopolysaccharides from periodontal pathogens in a high-fat environment to induce cell models of periodontitis and obesity. The transforming growth factor-β/mothers against decapentaplegic homolog (Smad) (TGF-β/Smad) pathway was evaluated both *in vivo* and *in vitro*. The indicators of renal function, renal pathological changes, and serum inflammatory cytokines were measured. The viability/apoptosis of RTECs and the expression of inflammatory cytokines were determined. Results: Periodontitis resulted in an increase in TGF-β/Smad activity in the kidney of obese mice. Moreover, the activity of RTECs was also increased *in vitro*. Downregulation of TGF-β led to reduced TGF-β, p-Smad2, p-Smad3, and Smad7 levels in kidney tissue and RTECs, ameliorated renal function indicators and renal pathological changes, increased viability and apoptosis of RTECs, and decreased levels of inflammatory cytokines. Conclusion: Periodontitis regulates renal impairment via the TGF-β/Smad pathway in obese mice.

Keywords: Obesity, renal impairment, periodontitis, TGF-β/Smad pathway

Introduction

Obesity is a major health threat in both developing and developed countries [1], which increases the risk of type 2 diabetes, hypertension, and osteoarthritis [2]. Periodontitis is attributed to the interaction between the gingival microbial community and innate and adaptive immunity [3], with the clinical presentations of periodontal inflammation, tooth loss, and pain [4]. In the progression of periodontitis, local gingival inflammation induced by malnourished microbial community and host immune over-reaction triggered by a large number of cytokines results in osteoclast activation and soft and hard tissue destruction [5]. Accumulative studies have reported an association between obesity and periodontitis [6-9]. Moreover, there is evidence showing that interleukin-6 (IL-6) and other pro-inflammatory cytokines are the key factors linking periodontitis and obesity. Obesity can easily lead to the long-term pro-inflammatory state of the human body, thereby disturbing the steady state of periodontal tissue and providing the basis for colony growth and reproduction [10]. For the kidneys, obesity is not only a predisposing factor for chronic kidney diseases, but also accelerates the progress of existing kidney diseases [11]. Chopra et al. believe that periodontitis is associated with renal insufficiency and inflammation [12]. Therefore, we speculated that periodontitis is a potential risk factor for obesity-related renal damage. Tumor necrosis factor-α (TNF-α) and IL-6 are common markers of inflammation, which play a vital role in immunoregulation [13]. Monocyte chemoattractant protein-1 (MCP-1) has been found to activate macrophages, as well as regulate renal inflammatory response [14]. With TNF-α, IL-6, and MCP-1 as critical indexes, the present research was designed to explore the effects of periodontitis on renal damage in obese mice.

Transcriptional growth factor-β (TGF-β) is widely involved in the biological processes of cell pro-

liferation, apoptosis, and differentiation, and it transmits information by activating mothers against decapentaplegic homolog (Smad) protein [15]. Therefore, the TGF-β/Smad pathway is a critical regulatory pathway for cell processes and disease progression. Kim et al. proposed that the TGF-β/Smad pathway mediated the senescence of human skin fibroblasts [16]. Also, it plays a pivotal role in obesity by regulating mesenchymal stem cells and adipocyte differentiation [17]. Moreover, the TGF-β/Smad pathway is able to affect renal fibrosis by regulating extracellular matrix, matrix metalloproteinase, and epithelial-mesenchymal transition [18]. Therefore, this pathway might be associated with obesity with impaired kidney function.

Although there is an inextricable relationship between periodontitis and obesity-induced renal damage, the underlying molecular mechanism remains elusive. In this study, we constructed mouse models of periodontitis and obesity to observe the changes in renal function and the TGF-β/Smad pathway, aiming to identify the potential mechanism underlying the regulation of periodontitis in obesity-related renal damage.

Methods

In vivo models of periodontitis complicated with obesity

Animals were treated on the basis of Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. Thirty six C57BL/6J mice (8-10 weeks, 246±9 g, Nanjing Institute of Biomedical Sciences) were raised under the suitable environment (room temperature: 25°C, relative humidity: 65% and 12/12-h light dark cycle), with food and water available *ad libitum*. *In vivo* models of periodontitis complicated with obesity were established with silk ligatures with bacteria in obese mice. Namely, mice were fed with a high-fat (60 kcal%) (n=24) and a normal diet (n=12) for 20 weeks [19]. At the 19th week, mice in the high-fat group (n=18) had their left maxillary second molar tooth ligated to induce periodontitis [20]. Thirty six mice were assigned into the control group (n=6, no treatment), peridontitis group (n=6, mice induced periodontitis), obesity group (n=6, high-fat diet), model group (n=6, high-fatdiet induced periodontitis), model + NC siRNA group (n=6, high-fat diet induced periodontitis and lentivirus infection containing NC siRNA), and model + TGF-β siRNA group (n=6, high-fat diet induced periodontitis and lentivirus infection containing TGF-β siRNA). Transfection protocol: Block-it Lentiviral RNAi Expression System (Invitrogen) was applied to encapsulate TGF-β siRNA (NC siRNA) into the lentivirus. Mice were anesthetized with 60 mg/kg pentobarbital, and lentivirus particles (1×10^4 transducing units per mouse) were injected into the caudal vein through a stereotactic device using a Hamilton syringe. TGF-β siRNA (NC siRNA) was designed and synthesized by Sangon Biotech Co., Ltd., Shanghai, China. Basic physical changes in mice were examined. Animal use and all experimental procedures were performed on the basis of management and use of experimental animals, and the present research was approved by the Ethics Committee of the Stomatological Hospital, Southern Medical University, with an ethics approval number of 2019-12-16.

In vitro renal damage models of periodontitis complicated with obesity

Renal tubular epithelial cells (RTECs) were obtained from the American type culture collection (ATCC, USA) and cultivated in a complete culture medium (DMEM + 10% FBS containing 100 U/mL penicillin and streptomycin) in a 5% CO_2, 37°C incubator. *In vitro* renal damage models of periodontitis complicated with obesity: first, RTECs were cultured in a complete culture medium (DMEM, Gibco, USA) containing 20 mg/L lysolecithin, and then treated with 100 μg/mL lipopolysaccharides (L2880, Sigma, USA) from periodontal pathogens. RTECs were assigned into the control group (no treatment), periodontitis group (incubated with lipopolysaccharides), obesity group (incubated with lysolecithin), model group (incubated with lysolecithin and lipopolysaccharides), model + NCsiRNA group (incubated with lysolecithin and lipopolysaccharides and transfected with liposome containing NC siRNA), and model + TGF-β siRNA group (incubated with lysolecithin and lipopolysaccharides and transfected with liposome containing TGF-β siRNA). The model + NC siRNA and model + TGF-β siRNA groups received transfection of NC siRNA and TGF-β siRNA (Sangon Biotech Co., Ltd., Shanghai,

Table 1. Primers for qRT-PCR

Gene	Direction of primer	Sequence (5' to 3')
TGF-β	Forward	GGCCAGATCCTGTCCAAGC
	Reverse	GTGGGTTTCCACCATTAGCAC
TNF-α	Forward	CCTCTCTCTAATCAGCCCTCTG
	Reverse	GAGGACCTGGGAGTAGATGAG
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTCAGGTTG
MCP-1	Forward	CAGCCAGATGCAATCAATGCC
	Reverse	TGGAATCCTGAACCCACTTCT
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

China), respectively, following the instructions of the Lipofectamine 2000 kit (Invitrogen). *In vitro* models were established in all groups except the control group.

Hematoxylin-eosin (HE) staining

After testing the basic physical changes, the mice were euthanized. Partial kidneys sampled from mice in each group were fixed in 10% neutral formalin. Subsequently, the samples were paraffin-embedded and sectioned, following by HE staining to observe the structure of renal tubules and glomeruli, as well as the arrangement of RTECs.

Cell apoptosis

The RTECs were cultured for 48 h after modeling. The apoptosis was determined by a FITC/Annexin V apoptosis detection kit II (BD Biosciences), and the apoptosis rate was analyzed using the CellQuest software (BD Biosciences).

Cell viability

An MTT Assay Kit (Solarbio) was used to determine cell viability. The cells in a 24-well plate were added with MTT reagent and allowed to stand for a period of time. Afterwards, optical density (OD) values at the peak wave length of 570 nm were read with a spectrophotometer, and the cell viability was evaluated.

Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was extracted from tissues or cells by Trizol to determine its purity. TGF-β, TNF-α, IL-6, and MCP-1 mRNAs were reverse-transcribed and amplified. All primer sequences were designed by Sangon Biotech Co., Ltd., Shanghai, China, and the reverse transcription and qPCR amplification kits were purchased from Solarbio. The composition of the reaction system and the setting of the reaction procedure were referenced from the product manual. The expression levels were calculated by $2^{-\Delta\Delta ct}$ after obtaining the Ct values of the samples. Primers are presented in **Table 1**.

Western blot

The cells were lysed (RIPA lysis buffer) and centrifuged for 20 min. The sediment was discarded, and protein concentration in the supernatant was quantified by the bicinchoninic acid (BCA) method (Thermo Fisher). Total protein extracted by SDS-PAGE (Solarbio) was blotted to a polyvinylidene fluoride membrane (Solarbio), and was allowed to react overnight with primary antibodies including TGF-β (1/1000, Abcam), p-Smad2 (1/1000, Abcam), p-Smad3 (1/2000, Abcam), Smad7 (1/3000, Abcam), TNF-α (1/1000, Abcam), IL-6 (1/1000, Abcam), MCP-1 (1/5000, Abcam), and GAPDH (1/2500, Abcam) against testing proteins and internal reference gene GAPDH (Abcam) at 4°C. Next, the protein was cultured with goat anti-rabbit secondary antibody at room temperature for 1 h, followed by the use of enhanced chemiluminescence (ECL) solution for visualization.

Statistical analysis

All data in the present research were analyzed using SPSS software (version 22.0; IBM Corporation). The data obtained from mouse experiments were all expressed in the form of mean ± standard error of the mean (SEM), and those from *in vitro* cell experiments were expressed as mean ± standard deviation (SD). The normal distribution of the data was verified by the K-S test. Data among multiple groups were compared by one-way ANOVA and Dunnett-t post hoc test, while normally distributed data were compared using the non-parametric test. The confidence interval used in this paper was 95%. $P<0.05$ was considered to indicate a statistically significant.

Results

Levels of TGF-β/Smad markers in periodontitis complicated with obesity

In vivo models of periodontitis complicated with obesity were established using silk ligatures with bacteria and high-fat diet, and *in vitro*

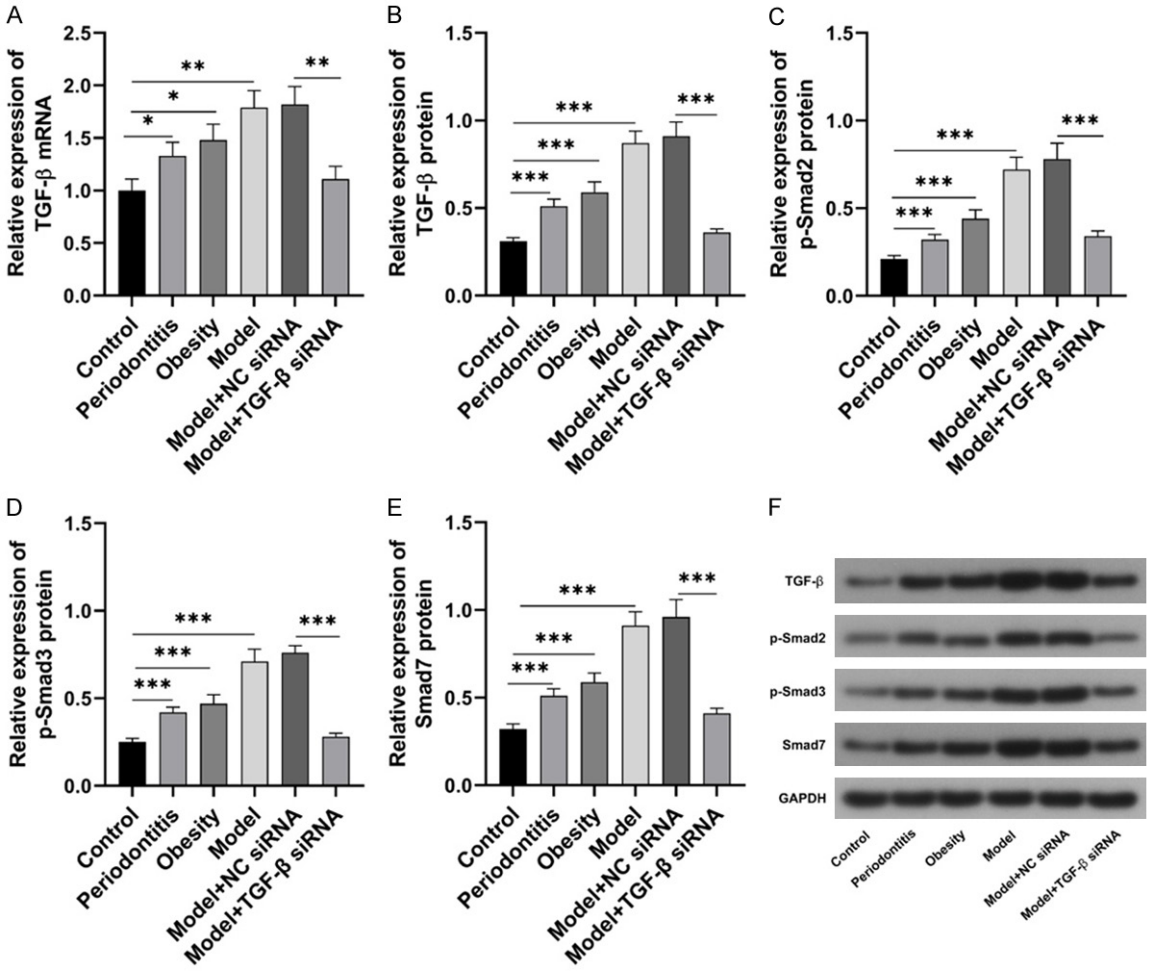


Figure 1. Levels of TGF-β/Smad markers in periodontitis complicated with obesity. *In vivo* models of periodontitis complicated with obesity were established using silk ligatures with bacteria and high fat diet (n=10 for each group). A, B. Periodontitis complicated with obesity induced the upregulation of protein and mRNA levels of TGF-β in RTECs, while TGF-β siRNA downregulated TGF-β levels. C. Periodontitis complicated with obesity stimulated Smad2 phosphorylation (p-Smad2), and downregulating TGF-β inhibited p-Smad2 levels. D. Periodontitis complicated with obesity stimulated Smad3 phosphorylation (p-Smad3), and downregulating TGF-β inhibited p-Smad3 levels. E. Periodontitis complicated with obesity induced an increase in Smad7 levels, and downregulating TGF-β inhibited Smad7 levels. F. Western blots. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

RTEC models were established with lipopolysaccharides from periodontal pathogens in a high-fat environment. The levels of TGF-β, p-Smad2, p-Smad3, and Smad7 in those models were measured.

As illustrated in **Figure 1**, periodontitis complicated with obesity upregulated the expression of TGF-β mRNA expression, which led to increased TGF-β and Smad7 protein expression and elevated phosphorylation of Smad2/3; however, downregulating TGF-β inhibited Smad2 and Smad3 phosphorylation and reduced protein levels of Smad7 *in vivo*. *In vitro* results in **Figure 2** shows the trends similar to **Figure 1**. We assumed that the TGF-β/Smad pathway might be the mediator of periodontitis complicated with obesity on renal function.

Influences of periodontitis on obesity via TGF-β/Smad pathway in vitro

In this paper, RTECs were treated with lipopolysaccharides from periodontal pathogens in a high-fat environment to induce cell models of periodontitis and obesity with renal damage. The regulatory role of the TGF-β/Smad pathway in the progression of periodontitis complicated with obesity was evaluated from three aspects of apoptosis, viability, and inflammatory cyto-

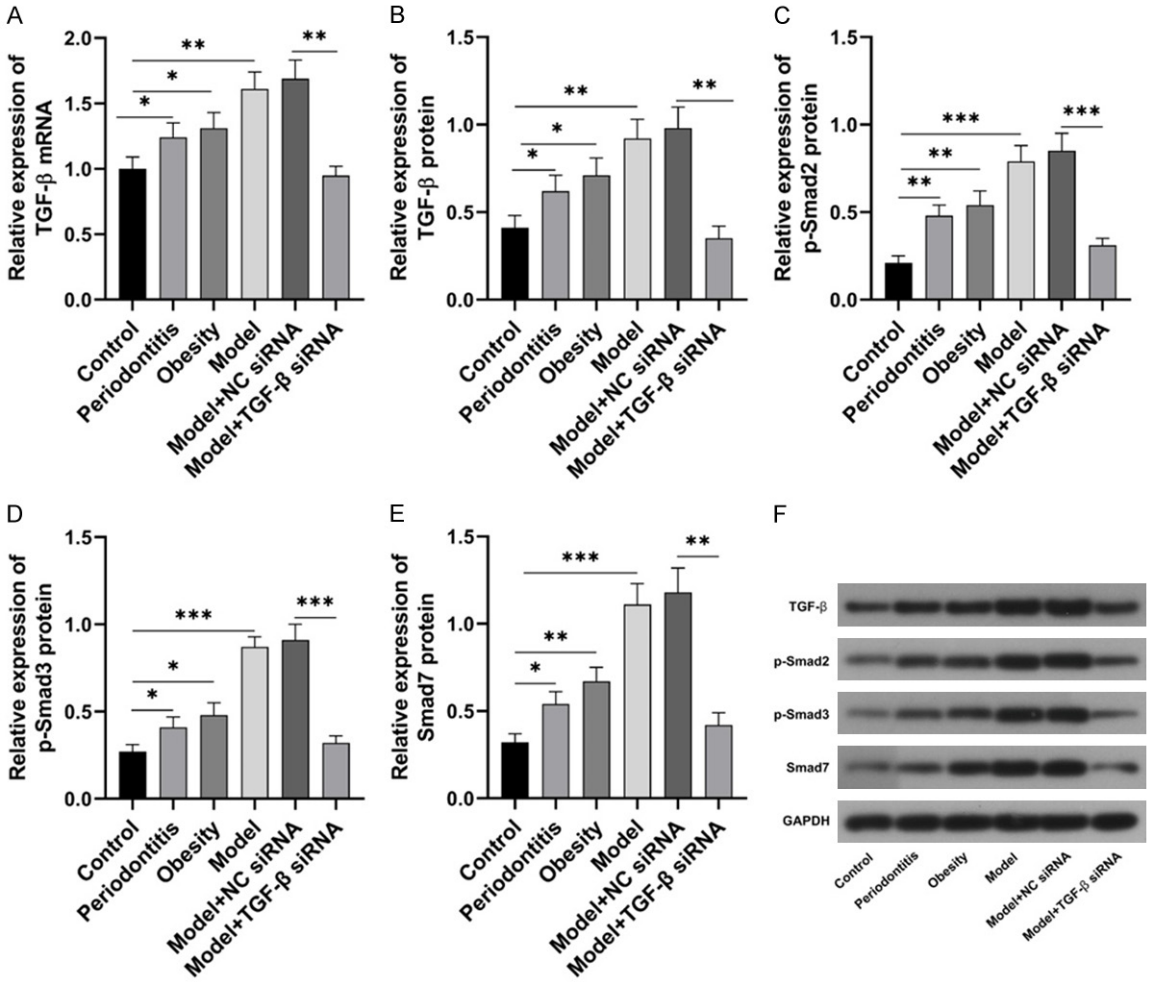
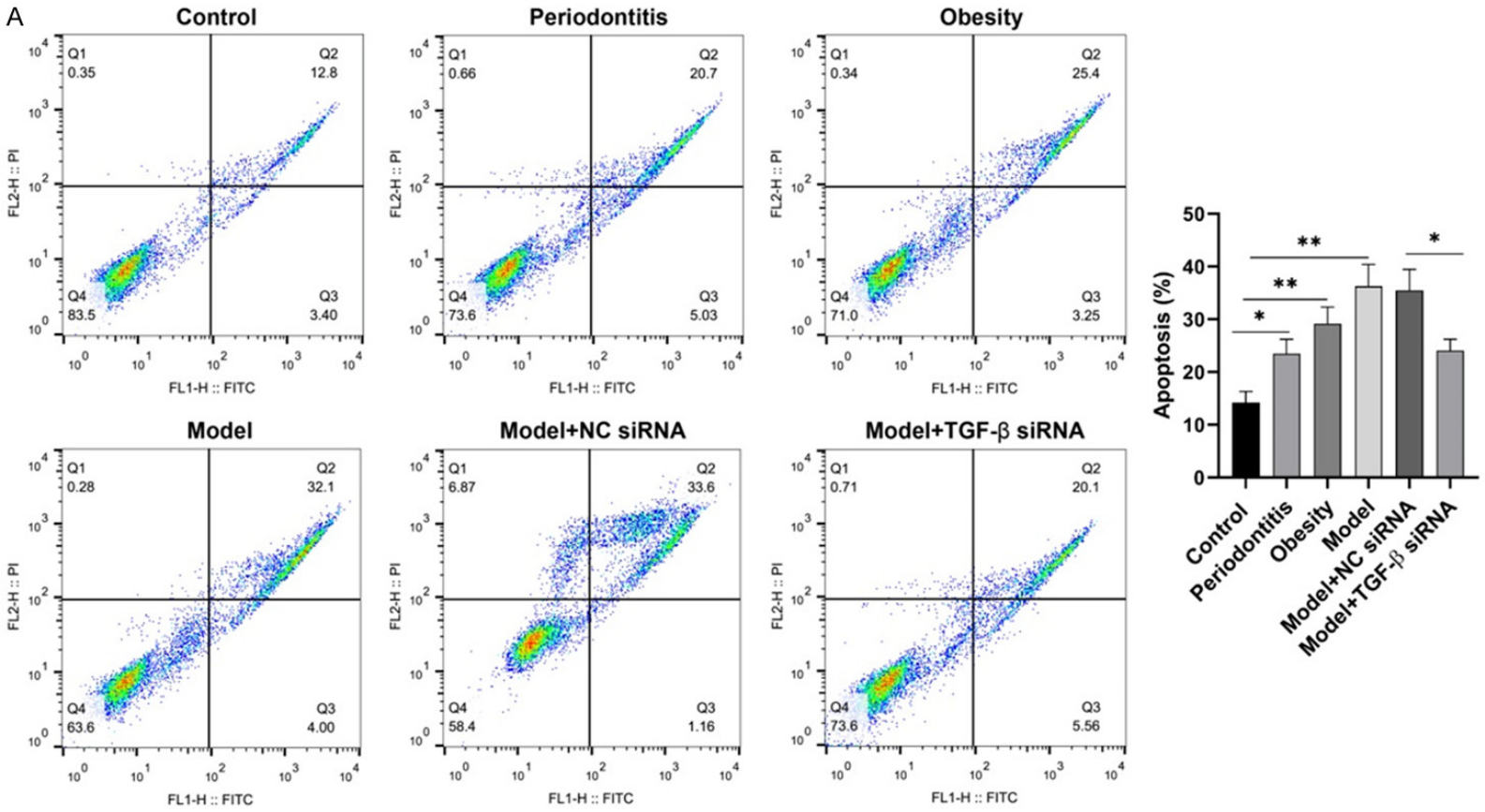


Figure 2. Levels of TGF-β/Smad markers in periodontitis complicated with obesity *in vitro*. RTECs were treated with lipopolysaccharides from periodontal pathogens in a high fat environment to induce cell models of periodontitis and obesity. A, B. Periodontitis complicated with obesity induced the upregulation of protein and mRNA levels of TGF-β in RTECs, while TGF-β siRNA downregulated TGF-β levels. C. Periodontitis complicated with obesity stimulated Smad2 phosphorylation (p-Smad2), and downregulating TGF-β inhibited p-Smad2 levels. D. Periodontitis complicated with obesity stimulated Smad3 phosphorylation (p-Smad3), and downregulating TGF-β inhibited p-Smad3 levels. E. Periodontitis complicated with obesity induced an increase in Smad7 levels, and downregulating TGF-β inhibited smad7 levels. F. Western blots. $^{*}P<0.05$; $^{**}P<0.01$.

kines. **Figure 3A** suggests that periodontitis complicated with obesity caused the increase of apoptosis of RTECs, which was resumed by the downregulation of TGF-β. **Figure 3B-H** show that periodontitis complicated with obesity induced the increase of inflammatory cytokines including TNF-α, IL-6, and MCP-1 at both protein and mRNA levels, while that trend was offset by the downregulation of TGF-β. In **Figure 3I**, periodontitis complicated with obesity resulted in decreased cell viability of RTECs. It turned out that periodontitis complicated with obesity led to enhanced apoptosis, suppressed viability, and increased levels of inflammatory cytokines (TNF-α, IL-6 and MCP-1). However, downregulation of the TGF-β/Smad pathway counteracted those effects (**Figure 3**). Therefore, apoptosis and inflammation in RTECs were aggravated and viability was inhibited in periodontitis complicated with obesity, which were counteracted by downregulating the TGF-β/Smad pathway.

Influences of periodontitis complicated with obesity on renal function via TGF-β/Smad pathway in vivo

In vivo models of periodontitis complicated with obesity were established using silk ligatures with bacteria and high-fat diet. Basic


A
Control
Q1 0.35
Q2 12.8
Q4 83.5
Q3 3.40
Periodontitis
Q1 0.66
Q2 20.7
Q4 73.6
Q3 5.03
Obesity
Q1 0.34
Q2 25.4
Q4 71.0
Q3 3.25
Model
Q1 0.28
Q2 32.1
Q4 63.6
Q3 4.00
Model+NC siRNA
Q1 6.87
Q2 33.6
Q4 58.4
Q3 1.16
Model+TGF-β siRNA
Q1 0.71
Q2 20.1
Q4 73.6
Q3 5.56
FL2-H :: PI
FL1-H :: FITC
Apoptosis (%)
0
10
20
30
40
50
*
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Control
Periodontitis
Obesity
Model
Model+NC siRNA
Model+TGF-β siRNA

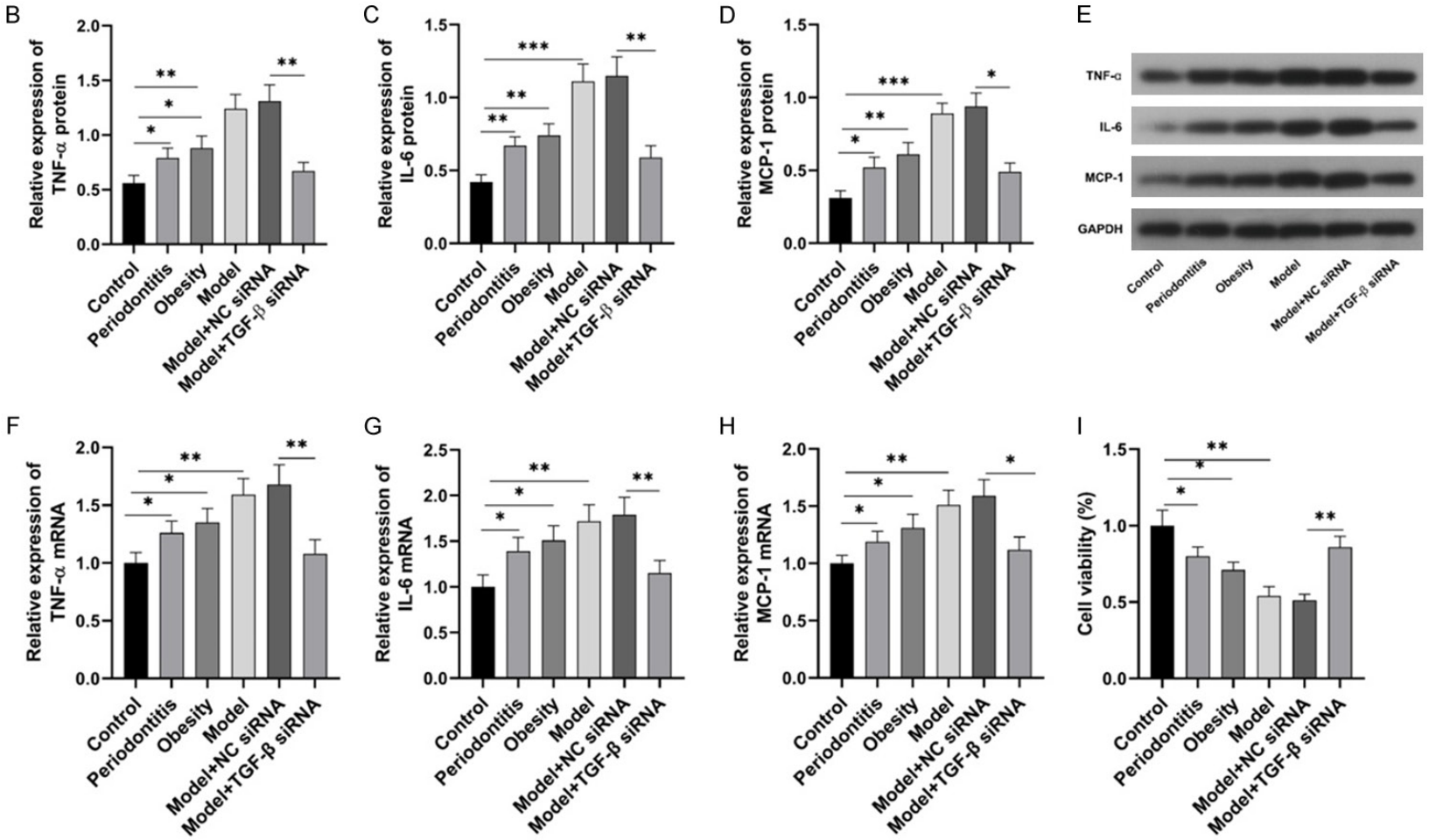

Figure 3. Regulatory role of TGF-β/Smad pathway in the progression of periodontitis complicated with obesity *in vitro*. RTECs were treated with lipopolysaccharides from periodontal pathogens in a high fat environment to induce cell models of periodontitis and obesity. A. Periodontitis complicated with obesity promoted apoptosis in RTECs, and downregulating TGF-β inhibited the apoptosis. B-H. Periodontitis complicated with obesity elevated the protein and mRNA levels of inflammatory cytokines (TNF-α, IL-6 and MCP-1), and downregulating TGF-β inhibited those levels. I. Periodontitis complicated with obesity restricted the viability in RTECs, and downregulating TGF-β enhanced the viability. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

physical changes in regard to lipid metabolism and renal function including body mass (BM), kidney mass (KM), total cholesterol (TC), free fatty acid (FTA), serum creatinine (Scr), serum cystatin-C (Cys-C), kidney injury molecule-1 (KIM-1), blood urea nitrogen (BUN), and Urinary creatinine (Ucr) were recorded, and the pathological changes in the kidney were observed by HE staining. In addition, TGF-β siRNA was injected into the renal cortical to suppress TGF-β in mice.

Periodontitis complicated with obesity induced the expression of BM, KM, Scr, Cys-C, and KIM-1, resulting in renal dysfunction. No significant change of BUN and Ucr level but an up-regulated trend was observed in the model group. Since the concentration of Ucr and BUN was highly variable in measurement, we calculated the value of Scr/Ucr and BUN/Scr (better for assessing kidney function) and found a significant elevation in the model group; the down-regulation of the TGF-β/Smad pathway decreased BM, KM, Scr, Cys-C, KIM-1, Scr/Ucr, and BUN/Scr (**Figure 4**). In control group, structures of renal tubules and glomeruli were clear, with orderly arranged RTECs. While periodontitis complicated obesity led to an increase in glomerular volume, unclear glomerular structure, smaller Bowman's capsule, and glomerulosclerosis. Moreover, RTECs swelled into vacuoles and showed dilatation of the lumen. After the downregulation of the TGF-β/Smad pathway, the kidney structure improved evidently, the glomerular volume decreased, Bowman's lumen and glomerular structure were clear, and RTECs recovered their orderly arrangement (**Figure 5**).

Influences of periodontitis complicated with obesity on inflammation via TGF-β/Smad pathway in vivo

As shown in **Figure 6A-C**, periodontitis complicated with obesity increased the levels of inflammatory cytokines (TNF-α, IL-6, and MCP-1) in serum. Meanwhile, western blot showed that the expression of TNF-α, IL-6, and MCP-1 was upregulated in renal tissue of rats with periodontitis complicated with obesity (**Figure 6D-G**). These results indicated that periodontitis complicated with obesity contributed to the inflammatory response in mice, which could be alleviated by down-regulating the TGF-β/Smad pathway.

Discussion

The incidence of overweight and obesity has greatly increased from 1980 to 2015, with one-third of the global population confirmed to be overweight or obese [21]. Patients with obesity are more susceptible to complications and in the meantime other adverse symptoms may occur more frequently.

There is a positive correlation between periodontitis and obesity [22]. A cross-sectional study indicates that there are significant statistical differences in the gingival index and community periodontal index between obese and non-obese groups [23]. In addition, a meta-analysis suggests that overweight and high body mass index (BMI) are risk factors for periodontitis [24]. Periodontitis and obesity have also been found to be interactively associated. Virto et al. believe that obesity complicated with periodontitis is responsible for the aggravation of systemic inflammation and metabolic abnormality, which simultaneously, leads to organ damage [25]. In the kidney, obesity destroys glomerular filtration through glomerular vessels, thereby elevating blood pressure and proteinuria [26]. Our findings demonstrated that obesity triggered impairment of renal function and pathological changes in the kidney, which was aggravated by periodontitis. Therefore, periodontitis is related to the aggravation of the impairment of renal function in patients with obesity. Obesity provides a necessary microenvironment for the development of periodontitis, while periodontitis increases abnormal renal metabolism and renal hemodynamic disturbance in obesity [27].

Why does periodontitis aggravate obesity-related renal damage? One of the reasons may lie in the upregulated TGF-β/Smad pathway in renal tissue and RTECs in periodontitis complicated with obesity. Renal fibrosis is a common manifestation of kidney diseases, and the TGF-β/Smad pathway plays an essential role in renal fibrosis [28, 29]. Zhang et al. confirm that the inhibition of TGF-β/Smad pathway protects against renal fibrosis in chronic kidney diseases [30]. Thus, we speculate that enhanced activity of this pathway leads to the increase of extracellular matrix accumulation and the decrease of its degradation in the kidney of patients with periodontitis and obesity results in renal impairment [31].

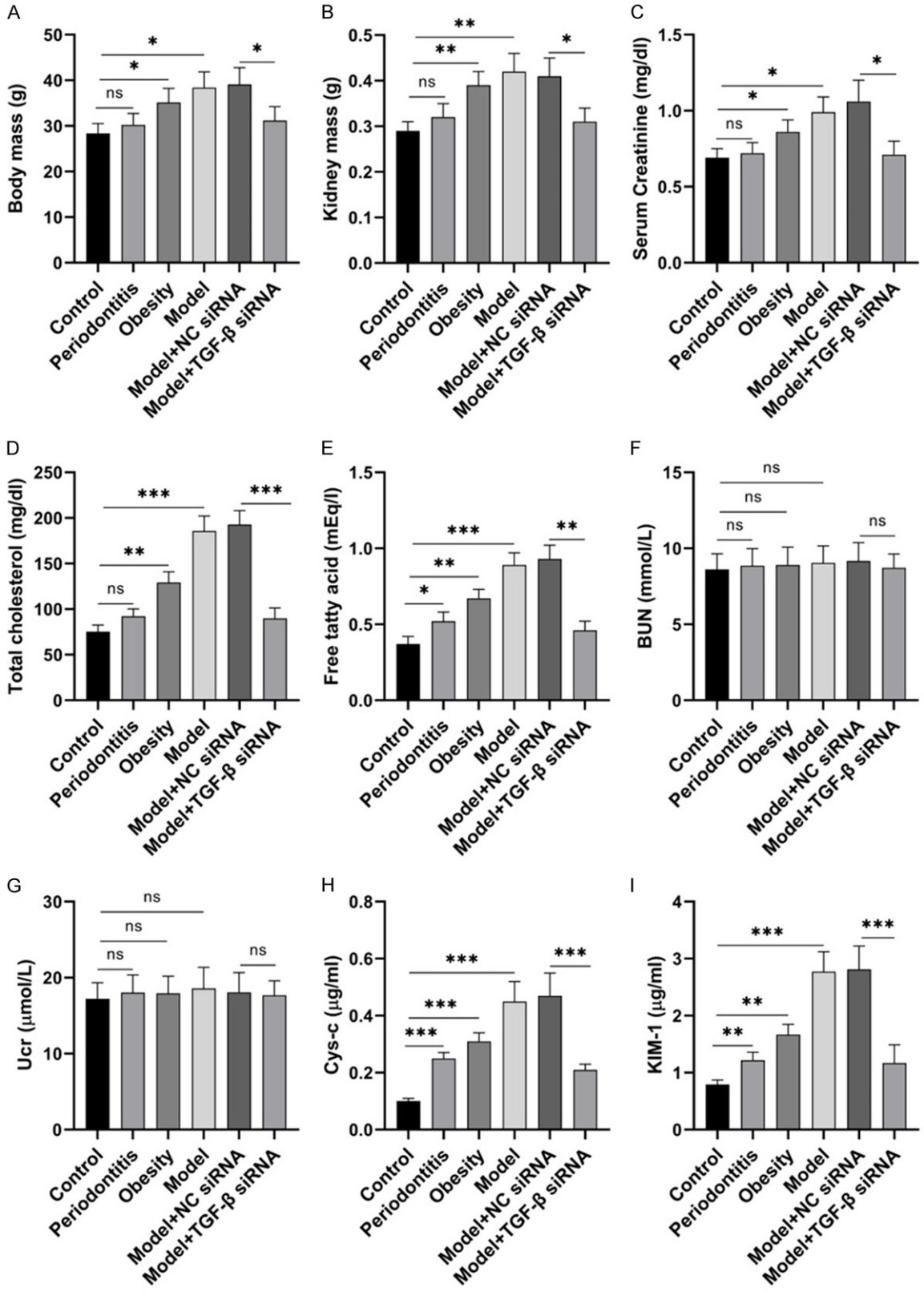

Figure 4. Basic physical changes of mice. A, B. Periodontitis complicated with obesity results in an increase in BM and KM, and down-regulation of TGF-β decreased both of them. C-I. Periodontitis complicated with obesity was associated with the increase in Scr, TC, FTA, Cys-C and KIM-1, but had little effect on BUN and Ucr. Downregulation of the TGF-β/Smad pathway ameliorated the above changes. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

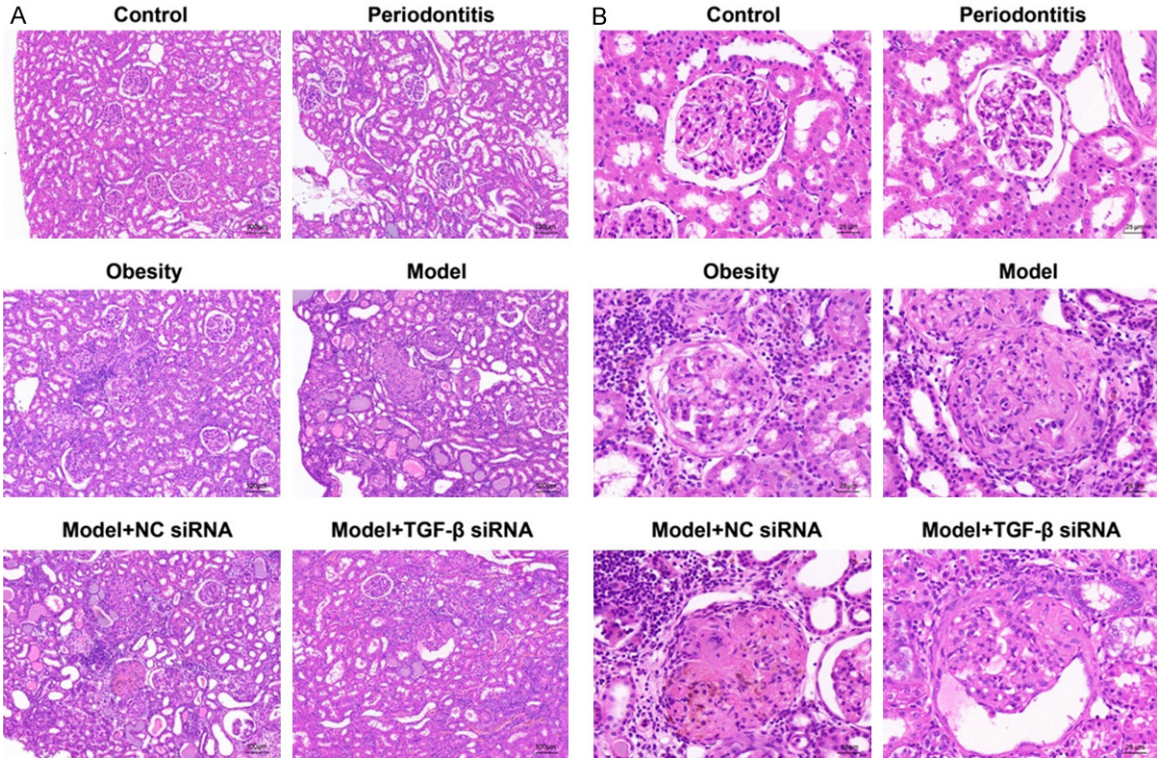

Figure 5. Measurement of renal pathological changes in each group (n=10) by HE staining. A. Renal sections were examined pathologically under 100× magnification; scale. B. Renal sections were examined pathologically under 400× magnification; scale. In control group, structures of renal tubules and glomeruli are clear, with orderly arranged RTECs. While periodontitis complicated with obesity led to an increase in glomerular volume, unclear glomerular structure, smaller Bowman's capsule and glomerulosclerosis; Moreover, RTECs swell into vacuoles and show dilatation of the lumen. After the TGF-β/Smad pathway was downregulated, the kidney structure improved evidently, the glomerular volume decreased, Bowman's lumen and glomerular structure were clear, and RTECs recovered their orderly arrangement.

Our findings in this study help to prevent or relieve the renal impairment induced by obesity. However, there are limitations as we did not monitor the renal function of obese patients with periodontitis, and analyze the risk index for periodontitis in obesity-related renal damage. In addition, other differentially expressed regulatory genes or signal pathways may be involved in the regulatory network of periodontitis in obese-related renal damage. The aggravated renal damage suggests that inhibiting periodontitis might promote obesity-related renal function recovery, which will be further elaborated in future studies. A prior study has pointed out [32] that, in the renal damage model of periodontitis complicated with obesity, the upregulation of the expression of TGF-β nucleic acid and protein in kidney tissue elevates the expression of Smad3 nucleic acid and p-Smad2/3 protein, which indicates that the activation of key signal molecules in the TGF-β/Smad signaling pathway promotes the transcription and protein expression of the corresponding target genes of TGF-β. After the treatment of Tripterygium wilfordii polyglycosides, the expression of TGF-nucleic acid and protein in kidney tissue was down-regulated, prior to the decreased expressions of Smad3 nucleic acid and p-Smad2/3 protein, indicating that tripterygium wilfordii polyglycosides interfere with the signal transduction of the TGF-β/Smad signaling pathway and antagonize the corresponding pathological effects of TGF-β. Therefore, Tripterygium wilfordii polyglycosides can inhibit the expression of key signal molecules in the TGF-β/Smad signaling pathway in the kidney tissue of model rats, interfere with the signal transduction of the TGF-β/Smad signaling pathway, regulate the expression of TGF-β, and improve renal damage.

To sum up, periodontitis regulates RTEC apoptosis and inflammatory cytokines through the

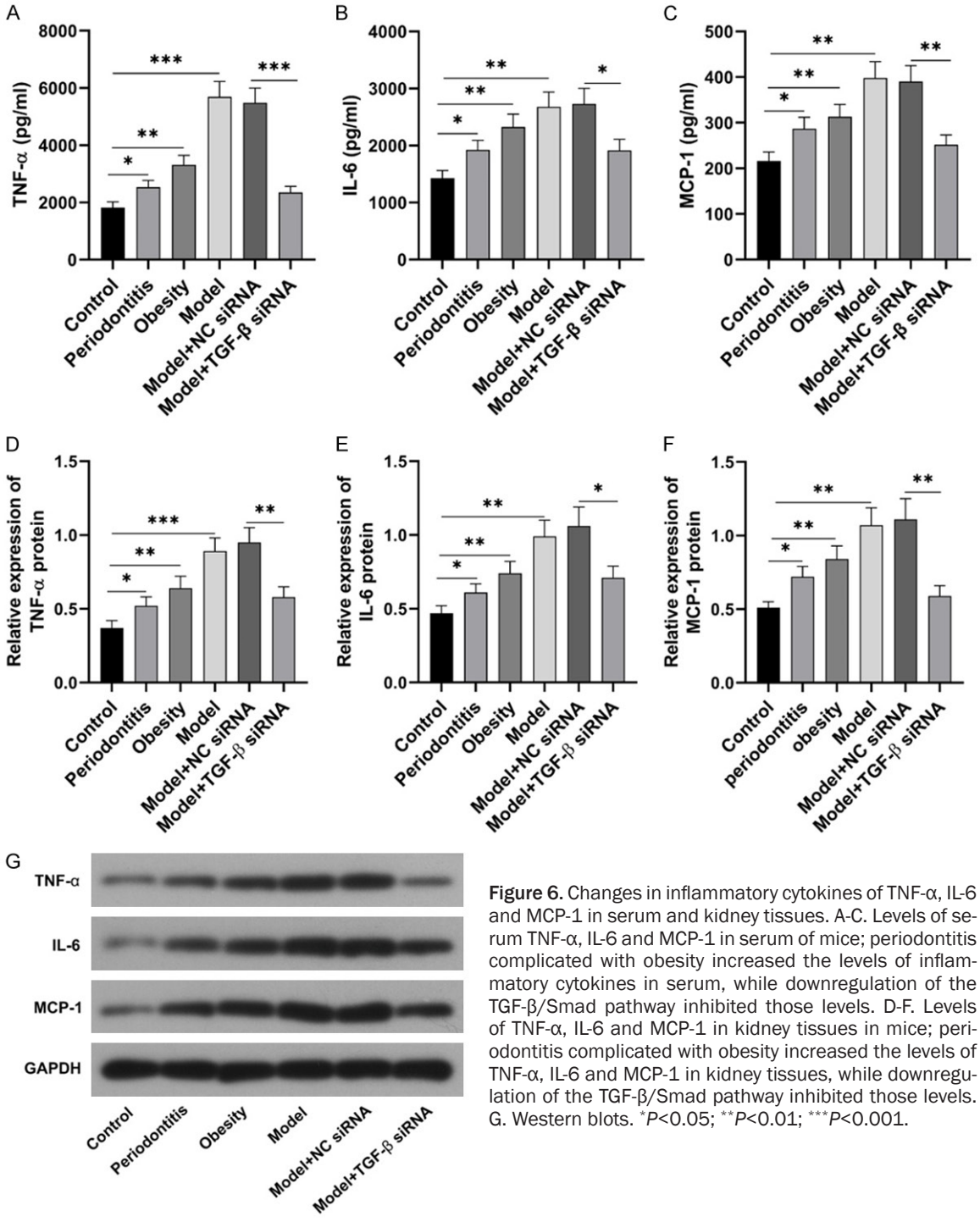

Figure 6. Changes in inflammatory cytokines of TNF-α, IL-6 and MCP-1 in serum and kidney tissues. A-C. Levels of serum TNF-α, IL-6 and MCP-1 in serum of mice; periodontitis complicated with obesity increased the levels of inflammatory cytokines in serum, while downregulation of the TGF-β/Smad pathway inhibited those levels. D-F. Levels of TNF-α, IL-6 and MCP-1 in kidney tissues in mice; periodontitis complicated with obesity increased the levels of TNF-α, IL-6 and MCP-1 in kidney tissues, while downregulation of the TGF-β/Smad pathway inhibited those levels. G. Western blots. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

TGF-β/Smad pathway, thus relieving the renal impairment induced by obesity. Moreover, down-regulation of TGF-β effectively restores the renal function in obese patients complicated with periodontitis. Therefore, monitoring the periodontal condition significantly contributes to the maintenance of stable renal function in obese patients.

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Disclosure of conflict of interest

None.

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