Original Research

Changes in advanced glycation end products, beta-defensin-3, and interleukin-17 during diabetic periodontitis development in rhesus monkeys

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Impact statement

The mechanism underlying the association between diabetes mellitus (DM) and periodontal disease is not yet fully understood. Hence, there is a need to establish animal models to reveal the effect of DM on the pathogenesis of periodontitis. In this study, we explored the appropriate methods for inducing periodontitis and shortening the modeling time in rhesus monkeys, to investigate the pathogenesis of diabetic periodontitis and develop innovative therapies. Our results suggest that a hyperglycemic environment might lead to the destruction of periodontal tissues by accelerating inflammatory response and weakening the defense system in periodontal tissues. Therefore, this study has significant treatment implications regarding the regulation of the immune response against periodontal diseases in patients with DM.

Abstract

The bidirectional relationship between diabetes mellitus (DM) and periodontal disease has drawn great attention; however, the mechanisms underlying their association remain unclear. In this study, we aimed to develop a rhesus monkey model of diabetic periodontitis and explore the potential mechanisms by which DM affects the progression of periodontal disease. Three healthy rhesus monkeys were selected as the control group. Five streptozotocin-induced diabetic rhesus monkeys were chosen as the experimental group. Ligature placement was used to induce periodontitis. The changes in the levels of advanced glycation end products (AGEs), beta-defensin-3 (BD-3), and interleukin-17 (IL-17) were measured using enzyme-linked immunosorbent assays (ELISA) and real-time reverse transcription polymerase chain reaction (RT-PCR) at different stages during disease progression. Periodontitis was confirmed by clinical assessment, radiographic images, and histological examination. Significant changes in the levels of AGEs and BD-3 in serum were observed at the periodontitis stage in diabetic rhesus monkeys ($P < 0.05$). The expression of BD-3 mRNA in the gingiva of diabetic group at baseline was significantly high ($P < 0.05$). Diabetic monkeys exhibited significantly enhanced IL-17 mRNA expression at the periodontitis stage ($P < 0.05$). Our findings indicated that the rhesus monkey can serve as an ideal model for exploring the pathogenesis of diabetic periodontitis, and the hyperglycemic environment may accelerate inflammatory response and weaken the defense system in periodontal tissues.

Keywords: Diabetes mellitus, periodontal disease, rhesus monkey, beta-defense-3, advanced glycation end products, interleukin-17

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that has a high incidence and prevalence worldwide. According to the International Diabetes Federation (IDF), the global prevalence of DM is approximately 8.8%, which is estimated to grow up to 10.4% by 2040.[1] DM is a risk factor for periodontitis,[2,3] but the potential mechanisms remain elusive. Periodontitis is a chronic, infectious disease caused by

periodontal pathogens, which may damage periodontal tissues directly or indirectly by stimulating host immune responses. Previous evidences indicate that patients with DM and their non-diabetic counterparts with similar severity of periodontal disease have comparable periodontal pathogen infection profiles.[4,5] Therefore, we speculate that the augmentation of host immune responses to periodontal pathogens, and impairment of defense system in periodontal tissues may be the main reasons behind the influence of DM on the progression of periodontitis, but not that of different subgingival microbiota. Previous data suggested that receptor of advanced glycation end products (RAGE) and its ligand, advanced glycation end products (AGEs), might partly mediate this pathological mechanism.[6,7]

Long-term hyperglycemia in diabetic hosts may exert acute and chronic effects on the body, for example, the formation of AGEs.[8] On one hand, AGEs exert their biological activity by binding to RAGE on cellular surfaces, followed by RAGE-mediated changes in signal transduction in the cells. On the other hand, AGEs can also function without RAGE. A number of previous studies have focused on RAGE, but very few studies have paid attention to the RAGE-independent biological role of AGEs.

The epithelium is the initial defense line against microbial invasion in periodontal tissues. A recent study showed that gingival epithelium also plays a crucial role in host innate immune response by secreting antibacterial peptides in addition to serving as a physical barrier. The results of previous studies have indicated that beta-defensin-3 (BD-3) is a salt-insensitive defensin, which makes it an important and a specific type of β-defensin (BD), because it can still exert its biological activity despite elevated salt concentrations in patients with systematic diseases that may inactivate the antimicrobial activity of other types of BD.[9] BD-3 can be induced by various stimulating factors, including bacteria and cytokines.[9–11] It is mainly located in the basal layer of healthy gingival epithelium.[12] However, the role of BD-3 in diabetic periodontitis remains undetermined to date, and inconclusive results have been reported regarded variation in its level during DM or periodontal disease.[9–11]

IL-17 is a major product of the newly discovered subset of helper T cells (T helper 17 [Th17]) and is multifunctional.[13] IL-17 secretion is affected by many cytokines. Currently, investigations on the mechanisms by which IL-17 influences the development and progression of diabetic periodontitis are limited, and there is a paucity of studies focusing on whether the expression of IL-17 is correlated with AGEs and BD-3 during the development and progression of diabetic periodontitis.

Previous researches investigating the mechanisms and interaction between DM and periodontitis have mostly been cross-sectional or *in vitro*. However, there is a lack of prospective studies. As cross-sectional or *in vitro* studies cannot simulate complex host responses, it is necessary and important to develop an animal model to mimic human diseases and explore the pathogenesis and correlation between DM and periodontitis.

Animals, such as mice,[14] rats,[15] rabbits,[16] dogs,[17] and pigs,[18] can be used to construct a disease model of periodontitis or diabetic periodontitis. These models can provide important data for investigating human diseases; nevertheless, it is difficult to determine whether these results can be applied to clinical treatment owing to species differences between these animals and human beings. Rhesus monkeys belong to *Macaca* and are the most commonly used non-human primates in pharmaceutical research. They are uniquely advantageous for biomedical studies because of their high genetic and organ structure similarity with humans.[19,20] There is evidence that rhesus monkeys can spontaneously develop periodontal disease.[21] However, wild rhesus monkeys exhibit a very slow natural developmental progression of periodontal disease and only mild gingivitis can be detected in most situations. Therefore, it is necessary to develop appropriate methods to induce diabetic periodontitis in rhesus monkeys that can exhibit alterations during disease development within a shorter time span and extrapolate these findings to humans to facilitate the investigation on the pathogenesis of diabetic periodontitis and development of innovative therapies.

In our study, we aimed to establish a disease model of diabetic periodontitis in rhesus monkeys to determine the levels of AGEs, BD-3, and IL-17 in serum and gingival tissues during the disease development process and analyzed further the correlation among the expression of these molecules. Through this study, we hope to determine whether DM would deteriorate periodontal disease by influencing host immune response and the defense system in periodontal tissues.

Materials and methods

Experimental animals

Eight male rhesus monkeys (age: 4–5 years; weight: 9–13 kg) were purchased from the Chengdu Ping'an Laboratory Animal Breeding Center (Chengdu, China). These animals were taken care of in accordance with the guidelines of the National Center for Safety Evaluation of Traditional Chinese Medicine (Chengdu, China), which has been approved by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Each monkey was fed in a separate cage. All the animals had free access to water and were fed a primate diet twice a day.

Establishment of disease model

Prior to the experiment, the rhesus monkeys were fed for two weeks to adapt to the environment. Then, they were divided into two groups: control group which comprised three healthy rhesus monkeys that were fed a normal diet, and experimental group which included five diabetic rhesus monkeys. The DM model was induced as described in a previous study.[22] Specifically, the animals were fasted for 16 h before modeling and injected intravenously with 100 mL of normal saline after anesthesia with ketamine (15 mg/kg intramuscular), followed by intravenous

injection of streptozotocin (STZ, 80 mg/kg). The fasting blood glucose (FBG) level was measured from the third day, and a FBG > 11.1 mmol/L for two consecutive days was indicative of successful diabetic modeling.[22] Diabetic rhesus monkeys were subcutaneously injected with porcine insulin (Wanbang Biopharma, Co. Ltd., Xuzhou, China) before feeding twice daily to maintain the blood glucose level at 6–10 mmol/L.

One year after successfully establishing the diabetic model, periodontal disease was induced in the experimental rhesus monkeys. After anesthesia with an appropriate amount of ketamine (15 mg/kg intramuscularly), tooth cervix in the left maxillary and mandibular first molars of diabetic rhesus monkeys were ligated with wires (0.2 mm in diameter). Meanwhile, the blood glucose level was maintained at 15–20 mmol/L by reducing the amount of insulin. The weights of these animals were determined regularly every month. Blood samples were collected to evaluate the routine blood and biochemical indicators.

Periodontal examination and histological examination of gingival biopsies

From the beginning of the study, all periodontal measurements were collected at baseline. The periodontal parameters were periodically recorded by a single calibrated operator once a month after wire ligation. The periodontal parameters reflecting the periodontal status are listed as follows: inspection of gingival color, form and contour; bleeding on probing (BOP); probing depths (PD); and attachment loss (AL). PD was defined as the distance from the free gingiva margin to the base of the gingival sulcus or periodontal pocket recorded to the nearest millimeter. AL indicates the distance between the cemento-enamel junction (CEJ) to the base of the probeable pocket. For each experimental tooth, clinical indices were detected at six sites using a periodontal probe, including mesiobuccal, buccal, distobuccal, mesiolingual lingual, and distolingual. The changes in periodontal parameters before and after modeling were compared to evaluate the severity of periodontitis.

Gingival samples were collected at baseline from the control and experimental groups and at the gingivitis phase and periodontitis stage from the experimental group. These gingival biopsies were fixed in 10% formalin, embedded in paraffin, and then sectioned for hematoxylin and eosin staining to observe inflammatory infiltration.

Imaging evaluation

Periapical radiographs of the teeth at the experimental sites and contralateral teeth were obtained using a distant paralleling technique to observe the distance from the alveolar ridge crest to the CEJ, i.e., the resorption of alveolar bone. Owing to the limitation of conventional two-dimensional radiographs, a cone beam computer tomography (CBCT) scan (Morita Manufacturing Corp., Kyoto, Japan) was also performed to determine the periodontal status of rhesus monkeys in a three-dimensional direction using 360° rotating projection. The operating parameters were as follows: 85 kV; 4.0 mA; exposure time, 17.5 s; slice thickness, 1.0 m; and spatial resolution of the reconstructed image, 125 μm. The software i-Dixel One Volume Viewer (Morita Manufacturing Corp., Kyoto, Japan) enabled the continuous observation of the distance from the alveolar ridge crest to the CEJ of the experimental and control teeth in different planes (axial, coronal, and sagittal).

Diagnosis of gingivitis and periodontitis

According to the diagnosis and classification of periodontal diseases and conditions proposed by the, 1999 International Workshop,[23] the diagnosis of gingivitis includes detection of slightly inflamed gingiva and BOP(+), but no detectable AL or bone loss, while the criteria of periodontitis comprise of inflamed gingiva, BOP(+), detectable AL, PD ≧ 5 mm in at least six sites, and vertical and horizontal bone resorption in radiographic images.

Blood collection

The weights of the animals were determined every month. Venous blood (2 mL) was collected, centrifuged, sealed in a dry aseptic Eppendorf tubes, and frozen at −80°C to determine the blood biochemical indicators.

Detection of the levels of AGEs and BD-3 in serum

The concentrations of AGEs and BD-3s were determined by AGEs ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA) and BD-3 ELISA kit of (Alpha Diagnostic Intl, Inc., San Antonio, TX, USA), respectively, according to the manufacturer's instructions. The minimum detection limits were as follows: AGEs, 0.39 μg/mL and BD-3, 1 pg/mL.

Detection of expression of BD-3 mRNA and IL-17 mRNA in gingiva

Quantitative RT-PCR was used to detect the expression of BD-3 mRNA and IL-17 mRNA in gingiva. Total RNA from gingival samples was extracted using TRIzol reagent (Invitrogen, USA), and subsequently reverse transcribed into cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, USA) to produce cDNA, which was subjected to quantitative PCR using the iQ SYBR Green Supermix (Bio-Rad Laboratories) with the iCycler iQ RT-PCR detection system (Bio-Rad Laboratories). All the primers were designed and generated by Shenggong Biotechnology (Shanghai, China). Detailed primer information is presented in Table 1. Each sample was tested in triplicates. The relative mRNA expression was determined as $2-\Delta\Delta CT$ and normalized to controls.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). ANOVA was used to compare the results between groups, and Spearman's rank correlation coefficient was employed for correlation analysis. All statistical analyses were performed using Graph prism (5.0) and $P < 0.05$ was considered statistically significant.

Table 1. Reverse transcription polymerase chain reaction primers used for RT-PCR.

Target	Primers (5′–3′)
BD-3	F: GGTCATGGAGGAATCATAAACA
	R: GGCATTTCCACACTTTACAACA
IL-17	F: CGC AAT GAG GAC CCT GAG AG
	R: GGA CCA GGA TCT CTT GCT GG
Actin	F: AGA AGC TGT CCT ATG TCG CC
	R: GCT CGT TGC CAA TGG TGA TG

Note: The total reaction system (20 μl in total) consisted of 10 μl fluorescence, 2 μl primer, 2 μl cDNA, and 6 μl without enzyme water.
RT-PCR: real-time reverse transcription polymerase chain reaction; BD-3: beta-defensin-3; IL-17: interleukin-17.

Table 2. Measurement of body weight and FBG of rhesus monkeys during diabetic periodontitis development.

		Diabetic group		
	Control group	Baseline	Four months	Nine months
Number	3	5	5	5
Age (years)	6.33 ± 0.94	6.80 ± 0.84	7.13 ± 0.84	7.55 ± 0.83
Body weight (kg)	7.69 ± 1.15	10.25 ± 2.17	10.08 ± 2.10	9.59 ± 3.09
FBG (mmol/L)	5.33 ± 1.03	6.42 ± 2.25	13.75 ± 6.56	23.47 ± 2.37

FBG: fasting blood glucose.

Results

Measurement of body weight and FBG of rhesus monkeys

Age, body weight, and FBG levels of the monkeys are presented in Table 2. At the baseline, the rhesus monkeys with diabetes were well matched with non-diabetic controls in terms of age, body weight, and FBG. After STZ injection and induction of DM, the average FBG level of rhesus monkeys in the experimental group increased progressively.

Periodontal examination

Diabetic monkeys and the non-diabetic controls had similar levels of dental plaque and dental calculus at baseline (Figure 1(a) and (b)). The control monkeys had pink and tough gingiva without BOP. Moreover, the PD was 2 mm and no AL was detected (Figure 1(a)), indicating that periodontal tissues in the controls were healthy. At baseline, diabetic monkeys exhibited tough gingiva without BOP or AL; however, a 2–3 mm PD and slight edema were detected (Figure 1(b)). Four months after wire ligation, the diabetic rhesus monkeys were found to have slightly red and swollen gingiva with BOP and a 3–4 mm PD, while no obvious AL was detected at the experimental sites. Nine months later, the rhesus monkeys in the diabetic group exhibited red and swollen gingiva, obvious BOP at the experimental sites, 4–7 mm deep periodontal pockets, along with AL at most sites (Figure 1(c)).

Table 3 presents the changes in PD values throughout the experiment period. The buccal PD values in diabetic monkeys were significantly greater than the values in control monkeys. As the disease developed, the value of PD in diabetic monkeys increased gradually, with greater value at nine months post-ligation than that at four months post-ligation and that at baseline.

Radiographic images

Nine months after ligature placement, we detected AL in diabetic monkeys. Therefore, two-dimensional X-ray measurement was performed to observe alveolar bone resorption (Figure 2). Furthermore, CBCT images were taken which enabled us to evaluate the extent of bone loss in three-dimensional view (Figure 3).

Figure 2 shows the visible bone loss on the mesial and distal crest of alveolar bone at experimental sites of diabetic monkeys at nine months compared to that at the control sites. Meanwhile, the sagittal views of CBCT scans revealed that the distance from CEJ to the mesial alveolar bone ridge in left maxillary first molar of diabetic monkeys (Figure 3 (d)) was dramatically greater compared with that in the control (Figure 3(a)). In addition, the periodontal membrane in the experimental tooth of diabetic monkeys (Figure 3(e)) was wider than that in the counterpart (Figure 3(b)). Moreover, the distance between CEJ and the labial crest of the alveolar bone in the left maxillary first molar of diabetic monkeys (Figure 3(f)) was greater than that in the control (Figure 3(c)).

Histological examination of gingival biopsies

Histology of gingiva samples revealed that there were more number of infiltrating inflammatory cells in diabetic rhesus monkeys at baseline (Figure 4(b)) than in the control group (Figure 4(a)). At four months post-ligation, gingivae of experimental monkeys displayed infiltrating inflammatory cells in connective tissues together with the extension of epithelial rete pegs (Figure 4(c)). Nine months after wire ligature, a greater number of inflammatory cells were observed in gingival tissues of diabetic rhesus monkeys, accompanied by edematous, deformed, and disintegrated collagen fibers beneath sulcular epithelium and junctional epithelium (Figure 4(c)), which is highly analogous to the histological characteristics of periodontitis in human beings.

According to the diagnosis and classification of periodontal diseases and conditions proposed by the 1999 International Workshop,[23] rhesus monkeys developed diabetic gingivitis at four months post-ligation and was diagnosed as diabetic periodontitis nine months after wire ligature based on the clinical and histologic features that were similar to gingivitis and periodontitis in humans. Thus, successful establishment of diabetic periodontitis at nine months post-ligation in rhesus monkeys was confirmed.

Detection of the level of AGEs and BD-3 in serum and the expressions of BD-3 mRNA and IL-17 mRNA in gingiva

The serum levels of AGEs and BD-3 and gingival expression of BD-3 mRNA and IL-17 mRNA are presented

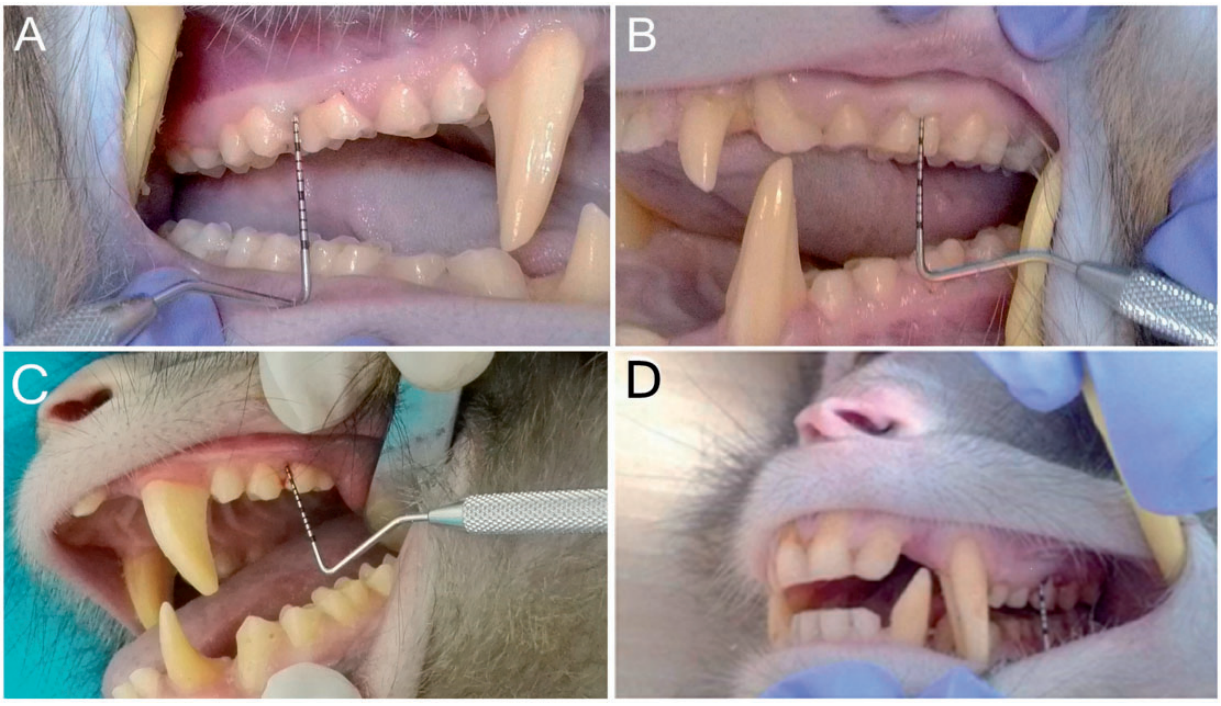

Figure 1. Periodontal examination of rhesus monkeys. (a) The control monkeys showed healthy periodontal tissues. (b) In the baseline stage, periodontal tissues of diabetic rhesus monkeys were basically healthy in the absence of BOP, AL or deep periodontal pocket (PD was 2–3 mm) except that their gingivae were a little bit edematous. (c) At 4-months post-ligation, the diabetic rhesus monkeys were found to have slightly red and swollen gingivae with BOP and a 3–4 mm PD, while no obvious AL was detected at the experimental sites. (d) Nine months after wire ligation, gingivae at experimental sites of these diabetic monkeys turned red and swollen which were more easily bleeding on probing; also, 4–7 mm deep periodontal pockets, along with AL at most sites were explored. BOP, bleeding on probing; AL, attachment loss; PD, probing depth. (A color version of this figure is available in the online journal.)

Table 3. Changes of PD values in rhesus monkeys during diabetic periodontitis development (mm).

		Diabetic group		
	Control group	Baseline	Four months	Nine months
Buccal PD	$1.28 \pm 0.58^{a,b,c}$	$2.22 \pm 0.67^{c,d}$	$2.47 \pm 0.78^{c,d}$	$3.00 \pm 1.41^{a,b,d}$
Lingual PD	$1.33 \pm 0.59^{b,c}$	1.90 ± 0.66^{c}	$2.17 \pm 0.75^{c,d}$	$2.78 \pm 1.90^{a,b,d}$

Note: Values are given as mean ± standard deviation.
PD: probing depth.
[a]Significantly different from baseline stage of diabetic rhesus monkeys ($P < 0.05$).
[b]Significantly different from gingivitis stage of diabetic rhesus monkeys ($P < 0.05$).
[c]Significantly different from periodontitis stage of diabetic rhesus monkeys ($P < 0.05$).
[d]Significantly different from control group ($P < 0.05$).

in Table 4. The serum levels of AGEs did not differ significantly between diabetic monkeys and healthy monkeys at baseline but they increased progressively with the exacerbation of periodontal disease in diabetic monkeys. At the periodontitis stage, diabetic monkeys exhibited significantly enhanced AGEs compared with the gingivitis and baseline phases. In contrast, the BD-3 level in the diabetic group was significantly higher than that in the controls at initial stage. While its content decreased gradually with the duration of periodontal disease, although not statistically significant.

Meanwhile, we observed that BD-3 mRNA expression in gingivae of diabetic group was higher than that in the non-diabetic control at baseline, and the expression gradually reduced, with lower levels at periodontitis and gingivitis stages than at the initial stage. The findings of this study showed that IL-17 mRNA expression increased as diabetic periodontitis developed. The IL-17 mRNA levels at the periodontitis stage in diabetic monkeys were markedly higher than the levels in the other groups. Additionally, diabetic monkeys exhibited enhanced gingival expression of IL-17 mRNA at gingivitis stage compared with the initial amount.

Correlation analysis of levels of FBG, AGEs, and BD-3 in serum, and expression of IL-17 mRNA and BD-3 mRNA in gingival tissues

The serum concentrations of AGEs were positively correlated with serum FBG levels and IL-17 mRNA expression in gingival tissues (Figure 5(a) and (b)). In addition, IL-17 mRNA expression in gingivae showed a negative correlation with the gingival expression of BD-3 mRNA (Figure 5 (c)), while the latter was consistent with serum BD-3 level (Figure 5(d)).

Discussion

The correlation between DM and periodontitis has been a concern recently, and periodontitis has been listed as the sixth complication of DM.[24] However, the potential

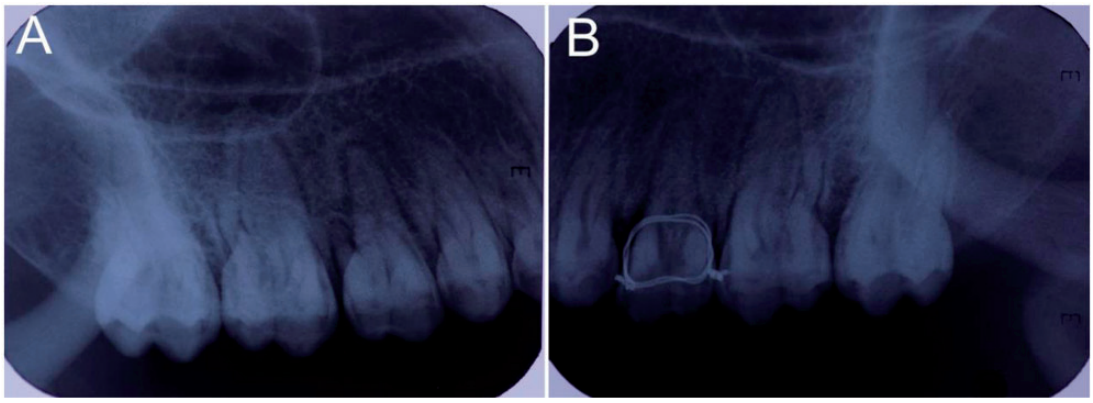

Figure 2. X-ray images of rhesus monkeys. (a) X-ray image of the control sites did not show obvious alveolar bone resorption, (b) while the radiographic image exhibited visible bone loss on the mesial and distal crest of alveolar bone at experimental sites of diabetic monkeys nine months after post-ligation.

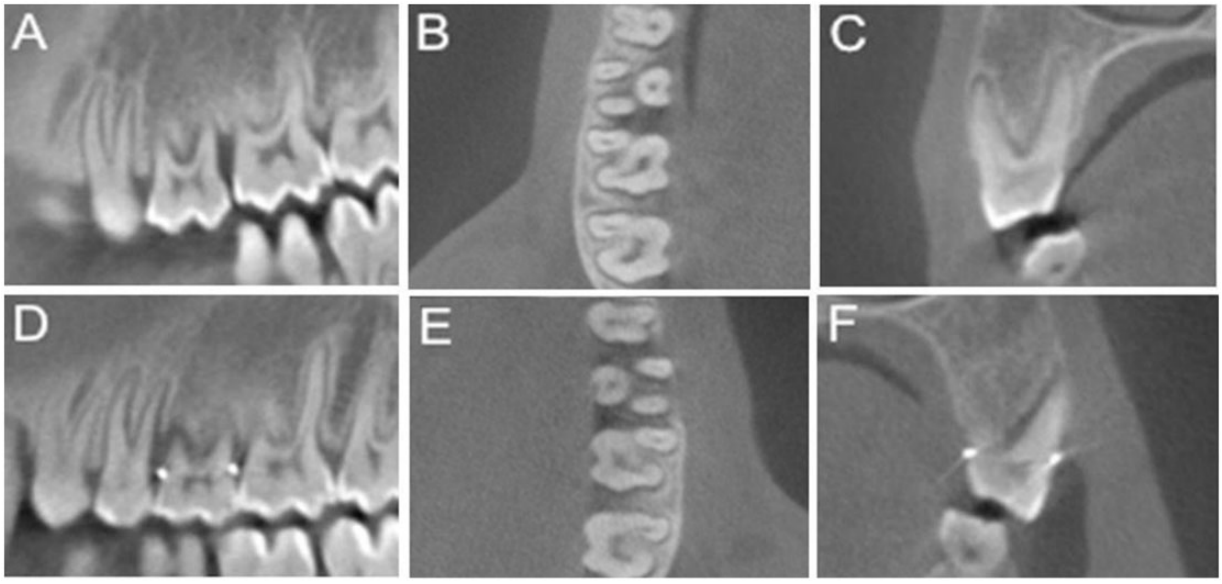

Figure 3. CBCT images of rhesus monkeys. The sagittal views of CBCT scans revealed that the distance from CEJ to the mesial crest of alveolar bone in left maxillary first molar of diabetic monkey (d) was dramatically greater compared with that in the control (a). Also, the horizontal section of CBCT images exhibited a wider periodontal membrane in the experimental tooth of diabetic monkey (e) than that in the counterpart (b). Moreover, the distance between CEJ and the labial alveolar ridge crest in left maxillary first molar of experimental monkey (f) was greater than that in the control (c).
CBCT, cone beam computer tomography; CEJ, cemento-enamel junctions.

mechanisms underlying their relationship have not been thoroughly investigated mainly because of the lack of an appropriate and reliable animal model for diabetic periodontitis. In this study, we constructed a prospective rhesus monkey model for diabetic periodontitis to explore the possible mechanisms by which DM affects periodontal disease.

The animals commonly used for the establishment of DM or periodontitis include mice, rats, rabbits, dogs, pigs, and non-human primates.[14–18] The incidence of periodontitis in rodents is low and the gingival sulcular epithelium is keratinized. Additionally, rodent teeth grow continuously, which is not helpful for the long-term histologic observations of periodontitis progression.[25–27] Although dogs have a higher incidence of periodontitis and show a gradually increased severity of this disease with age, the composition of oral bacterial communities and immune response of dogs are different from those of humans.[28] Miniature pigs develop periodontitis spontaneously, but the periodontal infection has a self-healing tendency.[29] Rhesus monkeys, which are non-human primates, resemble humans in phylogeny, oral cavity and tooth structure, oral flora, tartar, and plaque.[30] Moreover, non-human primates and humans exhibit similar immune responses to external pathogens.[31] Therefore, we hypothesized that rhesus monkeys can serve as an ideal animal model for investigations on diabetic periodontitis.

In the present study, the tooth cervix was ligated with wire to promote plaque accumulation and accelerate the progression of periodontitis, because previous studies have indicated that periodontitis develops very slowly in wild animals, generally only forming mild marginal gingivitis.[21]

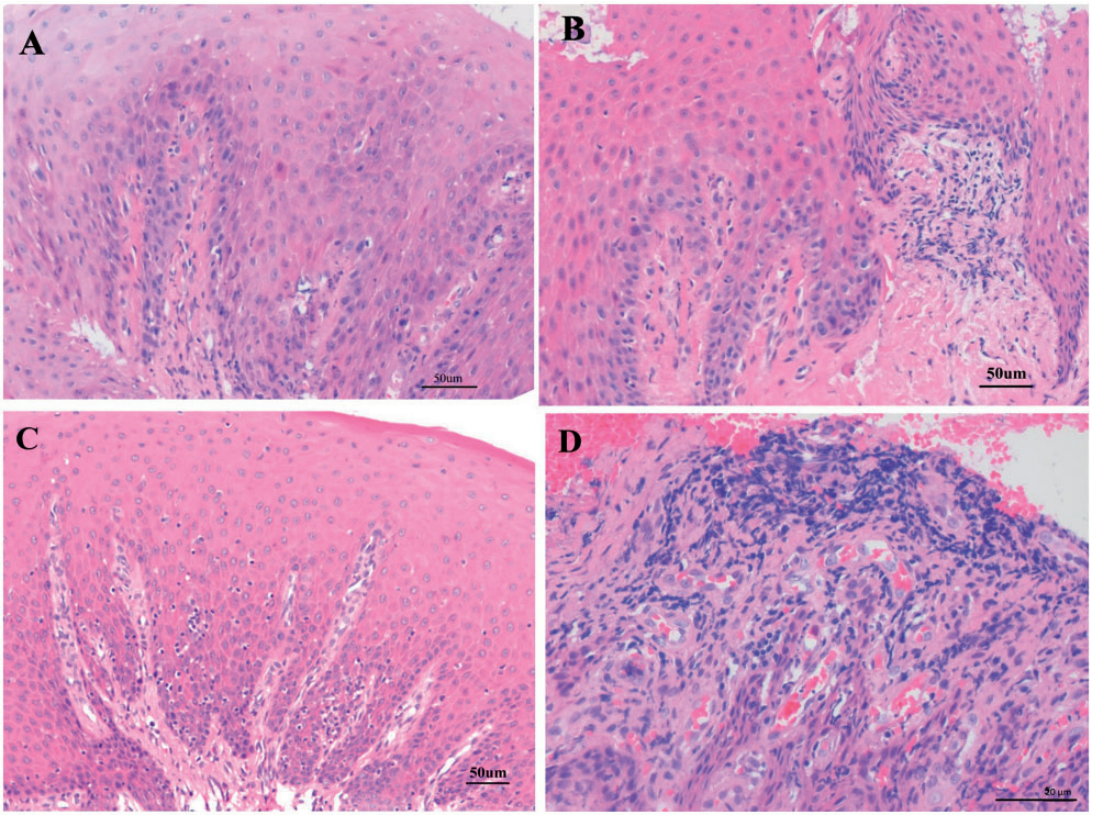

Figure 4. Histology of gingival biopsies (H&E stain). (a) The gingival tissues of control monkeys at baseline were normal. (b) Mild swelling of gingival epithelium combined with slight inflammatory infiltration was observed in gingival tissues from diabetic rhesus monkeys at baseline. (c) Four months after ligature placement, gingivae of experimental monkeys displayed infiltrating inflammatory cells in connective tissues together with the extension of epithelial rete pegs. (d) Nine months post-ligation, gingival tissues of diabetic monkeys exhibited infiltration of a greater number of inflammatory cells, such as lymphocytes, and showed that the collagen fibers beneath the sulcular and junctional epithelium were edematous, deformed, and disintegrated (Black bar: 50 μm). (A color version of this figure is available in the online journal.)

Table 4. Serum levels of AGEs, BD-3 and gingival expressions of BD-3 mRNA, IL-17 mRNA in during diabetic periodontitis development in rhesus monkeys.

		Diabetic group		
	Control group	Baseline	Four months	Nine months
AGE (μg/ml)	77.50 ± 9.7^{a}	80.29 ± 12.88^{a}	125.0 ± 19.55^{a}	$255.8 \pm 13.10^{b,c,d}$
BD-3 (pg/ml)	194.8 ± 29.8^{c}	2478 ± 564.70^{b}	1686 ± 186.90	1605 ± 164.00
BD-3 mRNA	1.00 ± 0.10^{c}	$13.32 \pm 4.79^{a,b,d}$	5.75 ± 1.72^{c}	2.93 ± 1.74^{c}
IL-17 mRNA	0.54 ± 0.23^{a}	$0.21 \pm 0.01^{a,d}$	$1.01 \pm 0.20^{a,c}$	$1.49 \pm 0.15^{b,c,d}$

Note: Values are given as mean ± standard deviation.
AGE: advanced glycation end products; BD-3: beta-defensin-3; IL-17: interleukin-17.
[a]Significantly different from periodontitis stage of diabetic rhesus monkeys ($P < 0.05$).
[b]Significantly different from control group ($P < 0.05$).
[c]Significantly different from baseline stage of diabetic rhesus monkeys ($P < 0.05$).
[d]Significantly different from gingivitis stage of diabetic rhesus monkeys ($P < 0.05$).

Four months after wire ligation, we observed that the periodontal status and histological features in experimental rhesus monkeys were similar to those in humans with gingivitis. At nine months post-ligation, diabetic monkeys exhibited periodontal inflammation, and histological observation closely resembled that for human beings.[32] Thus, the successful establishment of the diabetic periodontitis disease model in rhesus monkeys can be confirmed based on the inspection of gingivae, changes of BOP, PD, and AL, and examination of radiographic images and histology.

Notably, diabetic monkeys at baseline revealed slight gingival swelling compared with the controls, and inflammatory cell infiltration in gingivae of experimental monkeys was more severe than that of the control group at baseline, paralleling the more severe periodontal status in diabetic patients reported in previous studies.[33] Additionally, experimental monkeys exhibited deeper

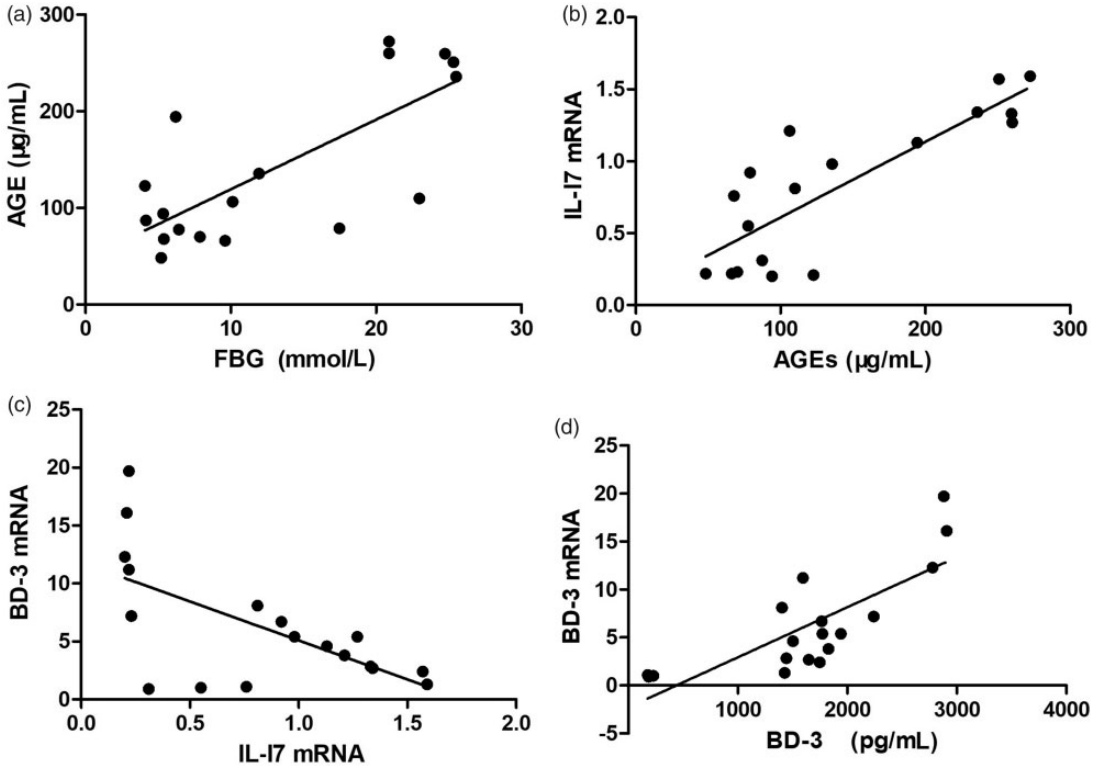

Figure 5. Correlation analysis of levels of FBG, AGEs, and BD-3 in serum, and expressions of IL-17 mRNA and BD-3 mRNA in gingival tissues. (a) The serum concentration of AGEs was positively correlated with serum FBG level. $R = 0.618$, $P < 0.05$. (b) The serum level of AGEs was consistent with IL-17 mRNA expression in gingival tissues. $R = 0.709$, $P < 0.05$. (c) The gingival expression of BD-3 mRNA was negatively related to the IL-17 mRNA expression in gingivae. $R = -0.574$, $P < 0.05$. (d) The correlation between the serum BD-3 level and gingival expression of BD-3 mRNA is positively related. $R = 0.750$, $P < 0.05$.
R, correlation coefficient; *P*, statistical significance. The correlation was significant at the 0.05 level. $^*P < 0.05$.

buccal PD than the controls at baseline which suggests poor periodontal conditions, indicating that DM may be a risk factor for periodontal disease. After wire ligation, the PD in diabetic monkeys deepened over time, which was indicative of progressive periodontitis in the experimental group.

Only 1–2 mm alveolar bone resorption was observed from CBCT images at nine months post-ligation compared with that at baseline in diabetic monkeys. Weinreb *et al.*[34] adopted the method of combining wire ligation with bacterial inoculation to induce experimental periodontitis in rhesus monkeys and found that there was obvious alveolar bone loss at some sites after four months. The application of wire ligation alone may be one of the reasons why it took a longer time to establish a disease model in our study. There are several common risk factors between DM and periodontitis, such as age, obesity, diet, and smoking.[35] In this study, we selected six- to eight-year-old rhesus monkeys (mean age, 6.80 ± 0.84 years), which is equivalent to 20–30 human years to rule out the impact of advanced age and possible confounding factors, such as smoking.[35] According to Persson *et al.*,[36] older rhesus monkeys (13–24 years) had a higher gingivitis index than young rhesus monkeys (4–5 years). Thus, we speculate that the young age of monkeys may also be responsible for the longer time for establishing the disease model in our study.

We discovered that the formation of AGEs in serum increased gradually as the disease developed and it was positively correlated with FBG levels, and these findings were consistent with the findings of Zizzi.[37] Recent evidences indicate that AGEs may contribute to periodontal tissue destruction by inhibiting the growth of human gingival fibroblasts (HGFs) or by stimulating the secretion of matrix matelloproteinase-1(MMP-1).[38,39] Whether AGEs influence periodontal disease by affecting the defense systems in periodontal tissues is not fully understood.

Defensins are antimicrobial peptides that are significant components of the innate immune system. Besides broad-spectrum antibacterial activity, BD-3 has many significant biological effects, such as influencing cytokine production and bridging innate and acquired immune responses.[40–42] BD-3 is induced by various stimulating factors, including bacteria and cytokines.[9–12] The results of our study showed that the serum level of BD-3 and gingival expression of BD-3 mRNA in diabetic monkeys were significantly higher than those in the controls at baseline, and their correlation was positive. Similarly, Bruno *et al.*, showed that the expression of BD-3 protein in patients with diabetic foot ulcer was higher than that in healthy people.[43] In contrast, Lan *et al.*, reported that the levels of BD-3 protein and BD-3 mRNA in the infected skin tissues of diabetic rats were significantly lower than the levels in the controls.[44] The differences in results obtained in different studies may be due to the different types of study models employed.

We speculated that the main reason behind these observations is the increased secretion of AGEs into the gingival crevicular fluid when the serum levels of AGEs increase

with diabetic periodontitis development.[45] AGEs usually form macromolecules on collagens. These macromolecules adhere to the peripheral walls of the veins and induce the thickening of the basal membrane and narrowing of the lumen of the vein, thereby, deteriorating the destruction of periodontal tissues. Furthermore, a high-glucose environment results in decreased chemotaxis and a reduced ability of polymorphonuclear neutrophils to degranulate.[46] Consequently, the defense ability of diabetic patients against microorganisms weakens, leading to an increase in microbes, which in return result in increased secretion of BD-3 by periodontal tissues to eliminate periodontal pathogens. Subsequently, as the disease developed, the expression of BD-3 mRNA in gingival samples decreased, and the expression was significantly lower in the diabetic periodontitis and gingivitis phases than the initial amount. This may be explained by the increased consumption of BD-3 to resist the invasion of bacteria and inhibit inflammation in periodontal tissues.

Previously, the Th1/Th2 paradigm was thought to be responsible for the pathogenesis of periodontitis. Recently, a new class of T cell subgroup (Th17 cell and its effector [IL-17]) has also been shown to be involved in the destruction of soft and hard tissues in periodontal disease by mediating activation of MMPs and enhancing osteoclast differentiation.[47–49] Our results showed that the expression of IL-17 mRNA increased gradually during the course of diabetic periodontitis, and were consistent with the values previously reported by Tomoyuki.[50] As a powerful pro-inflammatory cytokine,[51,52] IL-17 may aggravate and mediate the destruction of periodontal tissues in diabetic patients.

Correlation analyses revealed that the level of serum AGEs is positively correlated with the expression of IL-17 mRNA in gingiva, and the latter is negatively related to BD-3 mRNA expression in gingiva. We assume that the formation of AGEs is increased in a hyperglycemic state, which subsequently led to increased expression of IL-17 mRNA. Additionally, the expression of BD-3 mRNA reduced in gingiva. We hypothesize that the increased levels of AGEs may mediate the destruction of periodontal tissues by weakening the defense response of gingival tissues and by accelerating inflammatory response. However, the causal relationship among AGEs, IL-17 mRNA, and BD-3 mRNA needs to be validated further in future experiments.

However, several limitations of our study should be mentioned. Firstly, species differences exist despite the significant similarity of rhesus monkeys to humans in reflecting the clinical, histological, and molecular changes during disease development. Moreover, ethical reasons limit the generalization of this disease model to human beings. Finally, economic issues of buying and feeding primate animals limit the widespread application of this model in other research groups, and led to the limited sample size in our study which makes our experiment to be preliminary and needs to be validated using a larger sample size in future.

In summary, our study demonstrated that the rhesus monkey is an ideal animal for establishing a diabetic periodontitis disease model, and DM is a risk factor that accelerates the development of periodontitis. Additionally, our findings support the hypothesis that a hyperglycemic environment may result in periodontal tissue destruction by accelerating inflammatory response and weakening the defense system in periodontal tissues. These findings provide a mechanistic basis for a better understanding of how DM affects the development of periodontitis. Moreover, the results of this study have significant treatment implications in regulating the immune response of the host to periodontal diseases in patients with DM.

Author contributions: All authors participated in designing experiments, interpretation of the results, analysis of the data, and reviewing of the manuscript; HJ contributed to the experimental design, data acquisition, analysis, and interpretation, and drafted and critically revised the manuscript; PH substantially contributed to the conception and design of the study and finally approved the present version; YL assisted with the induction of diabetes in rhesus monkeys and finally proofread the manuscript; WW and CY critically revised the manuscript; YL and GL, contributed to data acquisition, analysis, and interpretation.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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