ORIGINAL ARTICLE

Therapeutic potential for insulin on type 1 diabetes-associated periodontitis: Analysis of experimental periodontitis in streptozotocin-induced diabetic rats

Toru Nishikawa[1], Yuki Suzuki[1], Noritaka Sawada[1], Yasuko Kobayashi[2], Nobuhisa Nakamura[2], Megumi Miyabe[2], Shin-ichi Miyajima[1], Kei Adachi[1], Tomomi Minato[3], Makoto Mizutani[4], Taku Toriumi[4], Norikazu Ohno[4], Takeshi Kikuchi[1], Masaki Honda[4], Toshihide Noguchi[1], Akio Mitani[1], Tatsuaki Matsubara[2], Keiko Naruse[2]* iD

[1]Department of Periodontology, School of Dentistry, Aichi Gakuin University, Nagoya, Japan, [2]Department of Internal Medicine, School of Dentistry, Aichi Gakuin University, Nagoya, Japan, [3]Department of Medical Technique, Aichi Gakuin University Dental Hospital, Nagoya, Japan, and [4]Department of Oral Anatomy, School of Dentistry, Aichi Gakuin University, Nagoya, Japan

ABSTRACT

Aims/Introduction: The association between diabetes and periodontal disease is considered to be bidirectional. However, there is still controversy surrounding the relationship between periodontal disease and type 1 diabetes. We investigated whether insulin improves periodontitis without any local treatments for periodontitis under type 1 diabetes conditions using the ligature-induced experimental periodontitis model.

Materials and Methods: Type 1 diabetic rats were induced by streptozotocin injection. Experimental periodontitis was induced by ligature in normal and diabetic rats. Half of the diabetic rats were treated with insulin. Two weeks after the ligature, periodontitis was evaluated.

Results: Insulin treatment significantly improved inflammatory cell infiltration and inflammatory cytokine gene expression, leading to suppression of alveolar bone loss, in the periodontitis of diabetic rats. Insulin also suppressed the periodontitis-increased nitric oxide synthase-positive cells in periodontal tissue of the diabetic rats. Even without induction of periodontitis, diabetic rats showed decreased gingival blood flow and an increased number of nitric oxide synthase-positive cells in the gingiva and alveolar bone loss compared with normal rats, all of which were ameliorated by insulin treatment. We further confirmed that insulin directly suppressed lipopolysaccharide-induced inflammatory cytokine expressions in THP-1 cells.

Conclusions: There were abnormalities of periodontal tissue even without the induction of periodontitis in streptozotocin-induced diabetic rats. Insulin treatment significantly ameliorated periodontitis without local periodontitis treatment in diabetic rats. These data suggest the therapeutic impacts of insulin on periodontitis in type 1 diabetes.

INTRODUCTION

Periodontitis is a chronic inflammation whereby pathogens in periodontal pockets destroy the gingiva, and subsequently, the alveolar bone is absorbed[1]. The induction and propagation of periodontitis are through a dental plaque, which interacts with the immune defenses of the host, leading to inflammation[1–3].

In diabetes patients, periodontal disease is considered to be one of the diabetic complications because of the high prevalence and severity[4,5]. The involvement of several biological abnormalities based on diabetes, such as immune response deficiency, vascular and bone abnormalities, and metabolic disorders, are considered[6,7]. As the interaction between diabetes and periodontal disease is considered to be bidirectional[8], the prevention of periodontal disease is crucial not only for oral health,

but also for the maintenance of good glycemic control in diabetes patients.

Epidemiological and clinical evidence for an association between periodontitis and diabetes is accumulating[9]. However, there is controversy regarding the relationship between periodontal disease and type 1 diabetes. Several epidemiological studies showed that type 1 diabetes patients with poor glycemic control have been demonstrated to have aggravated periodontal disease compared with patients with good glycemic control[10,11]. In contrast, a meta-analysis and recent cohort study did not show a significant difference of the periodontal status in non-diabetic controls and type 1 diabetes patients[12,13]. A systematic review showed a lack of sufficient evidence for the relationship between periodontitis and glycemic control in type 1 diabetes patients[9,14].

Ligature-induced experimental periodontitis progresses in a similar manner as human periodontal disease[15]. In the present study, we investigated whether the administration of insulin in the absence of local periodontal therapy improved ligature-induced experimental periodontitis in streptozotocin (STZ)-induced type 1 diabetic rats. We also confirmed that insulin directly suppressed the expression of inflammatory cytokines in a human monocyte/macrophage cell line, THP-1 cells.

METHODS

Animals and induction of diabetes
Male Sprague–Dawley rats (aged 5 weeks) were provided by Chubu Kagakushizai (Nagoya, Japan). Six-week-old Sprague–Dawley rats were weighed after the overnight fasting and STZ (60 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) was injected for the induction of diabetes. Rats with plasma glucose concentrations of >15 mmol/L were selected as the diabetic rats at 1 week after the STZ-injection. The experimental animal groups were as follows: normal rats, diabetic rats and diabetic rats with insulin treatment ($n = 20$ in each group). All rats were induced with experimental periodontitis on one side of the maxillary second molar (M2), as described below. During the experiments, one normal rat and two diabetic rats died and were excluded from the experiments. All experimental protocols were carried out according to the Regulations for Animal Experiments in Aichi Gakuin University, and were approved by the Institutional Animal Care and Use Committees of Aichi Gakuin University (AGUD-043).

Induction of periodontitis
Two weeks after the administration of STZ, a nylon ligature was tied around the M2 to induce periodontitis, as previously described[16]. The M2 on the other side remained without any treatment as the control side (Figure 1a).

Insulin treatment
Half of the diabetic rats were treated with insulin. Insulin treatment was carried out by the insertion of insulin pellets (Lin-Shin Canada Co., Toronto, ON, Canada; $n = 20$). We cut a
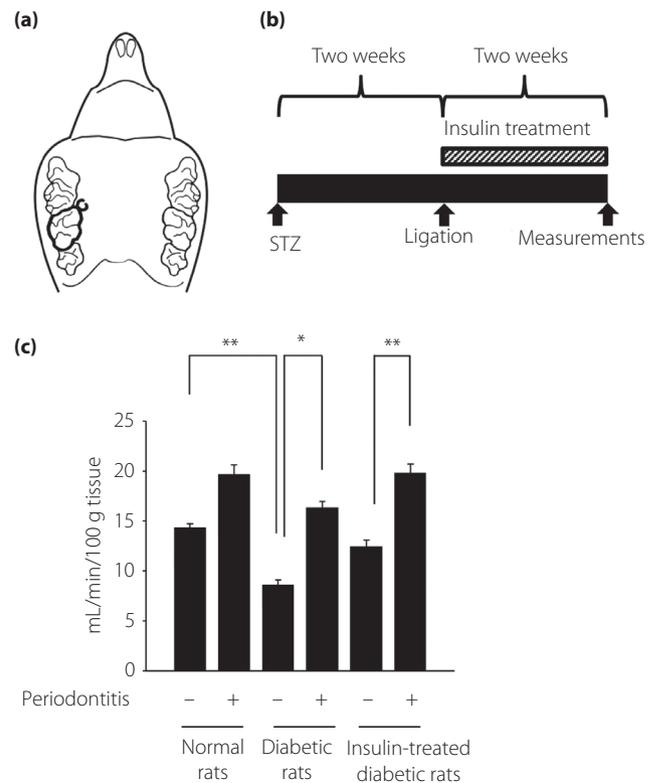

Figure 1 | (a) Ligature-induced periodontitis. Nylon thread was ligated around the unilateral maxillary second molar. The other side was untreated as the control. (b) Streptozotocin (STZ) was intraperitoneally injected to induce diabetes. Two weeks after STZ injection, experimental periodontitis was induced by ligation. Half of the diabetic rats received insulin treatment for 2 weeks. At 2 weeks post-surgery, physiological and pathological assessments were carried out. (c) Gingival blood flow of the buccal side above the maxillary second molar was measured using a laser Doppler blood flow meter. The results are expressed as the mean ± standard error of the mean ($n = 9$). *$P < 0.05$, ** $P < 0.01$.

small lesion in the back skin and inserted an insulin pellet that was left in for 2 weeks (Figure 1b). The effects of insulin were confirmed by reductions in blood glucose.

Blood flow in the gingival tissue
The blood flow in the gingival tissue was measured using a laser Doppler blood flow meter (FLO-N1; Omega Wave Inc., Tokyo, Japan). The rats ($n = 9$ in each group) were anesthetized by the intraperitoneal injection of a combination of medetomidine, midazolam and butorphanol. Rats were placed on a heated pad to keep a constant rectal temperature of 37°C. Blood flow on the buccal side of the gum of the M2 was measured.

Tissue collection
Two weeks after the ligation, rats were killed by an overdose of pentobarbital (1.5 mg/kg). For messenger ribonucleic acid

(mRNA) and protein analyses, gingival tissues were obtained and snap-frozen in liquid nitrogen and kept at −80 °C until use. For immunohistological and micro-computed tomography (CT) analyses, maxillary bones with gingiva on both sides were fixed in a 10% formalin solution.

Gene expression of gingiva

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and complementary deoxyribonucleic acid was synthesized using ReverTra Ace (Toyobo, Osaka, Japan). Primers and probes were purchased from Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Real-time quantitative polymerase chain reaction was carried out and measured by the ABI Prism 7000 (Applied Biosystems; $n = 8$ in each group). Relative quantity was calculated by the $\Delta\Delta C_t$ method using β_2 microglobulin as the endogenous control[17].

Histological evaluation

Fixed tissues were decalcified in 10% ethylenediaminetetraacetic acid for 5 weeks, dehydrated using a graded ethanol series and then embedded in paraffin. Specimens were sectioned (4-μm thick) and stained with hematoxylin–eosin ($n = 6$ in each group). For immunohistological staining, anti-inducible nitric oxide synthase (iNOS) antibody (Bioss Antibodies Inc., Woburn, MA, USA) was used for the first antibody and subsequently stained using Simplestain rat system (Nichirei, Tokyo, Japan; $n = 6$ in each group).

Micro-CT imaging

Maxillae were scanned by micro-CT (R_mCT; Rigaku Corporation, Tokyo, Japan; $n = 6$ in each group). The distance from the mesial buccal cement–enamel junction to the alveolar bone crest of the second molar was measured as a marker of bone height. The percentage of bone resorption was calculated as the distance from the mesial buccal cement–enamel junction to the alveolar bone crest/root length × 100.

Lipopolysaccharide-stimulated inflammatory response in THP-1 cells

A human monocyte/macrophage cell line, THP-1 cells, was purchased from American Type Culture Collection (Manassas, VA, USA). After the THP-1 cells were starved without serum for 24 h, human insulin (Sigma-Aldrich) was added at the concentration of 10 or 100 nmol/L 30 min before lipopolysaccharide (LPS) from Escherichia coli (Sigma-Aldrich) stimulation. LPS (100 ng/mL) was added and cells were collected 4 h later for total RNA extraction using RNeasy (Qiagen, Valencia, CA, USA).

Statistical analysis

All group values were expressed as the mean ± standard error of the mean. Statistical analyses were made by two-way ANOVA followed by the Bonferroni correction for multiple comparisons. Differences were considered significant at the $P < 0.05$ level.

RESULTS

Bodyweights, blood glucose and glycated hemoglobin

Diabetic rats showed significant weight loss, hyperglycemia and higher glycated hemoglobin compared with the normal rats ($P < 0.001$; Table 1). Insulin treatment of the diabetic rats significantly increased the bodyweight, and decreased blood glucose and glycated hemoglobin levels.

Gingival blood flow

On the control side, gingival blood flow in the diabetic rats was significantly lower compared with that in the normal rats ($P < 0.01$), which was ameliorated by insulin treatment (the control side of normal rats: 14.3 ± 0.4 mL/min/100 g, the control side of the diabetic rats: 8.6 ± 0.5 mL/min/100 g, the control side of the insulin-treated diabetic rats: 12.4 ± 0.5 mL/min/100 g; Figure 1c). The induction of periodontitis led to an increase in gingival blood flow. In the diabetic rats, the gingival blood flow of the periodontitis side was significantly increased by 1.9-fold from the control side. Insulin treatment tended to decrease the increased rate of blood flow by periodontitis in the diabetic rats (1.5-fold increase); however, the increase is still significant ($P < 0.01$).

mRNA expressions of the inflammatory cytokines in the gingiva

A small increase in the mRNA expression of tumor necrosis factor (TNF)-α and iNOS on the periodontitis side were shown in the normal rats, but they were not significant (Figure 2). In the diabetic rats, periodontitis induced significant increases in both TNF-α and iNOS mRNA expression compared with the expression on the control side ($P < 0.001$). The increase in TNF-α and iNOS mRNA expression induced by periodontitis was significantly greater in the diabetic rats compared with those in normal rats. Insulin treatment significantly suppressed the periodontitis-induced increase in TNF-α and iNOS mRNA expression in the diabetic rats ($P < 0.01$ and $P < 0.001$, respectively).

Table 1 | Characteristics of normal, diabetic and insulin-treated diabetic rats

	Bodyweight (g)	Blood glucose (mmol/L)	HbA1c (%)
Normal rats	323.8 ± 7.3**	6.4 ± 0.2**	4.0 ± 0.1**
Diabetic rats	192.8 ± 22.0	30.9 ± 1.8	7.1 ± 0.2
Insulin-treated diabetic rats	295.8 ± 18.5*	7.6 ± 0.6**	5.2 ± 0.2**

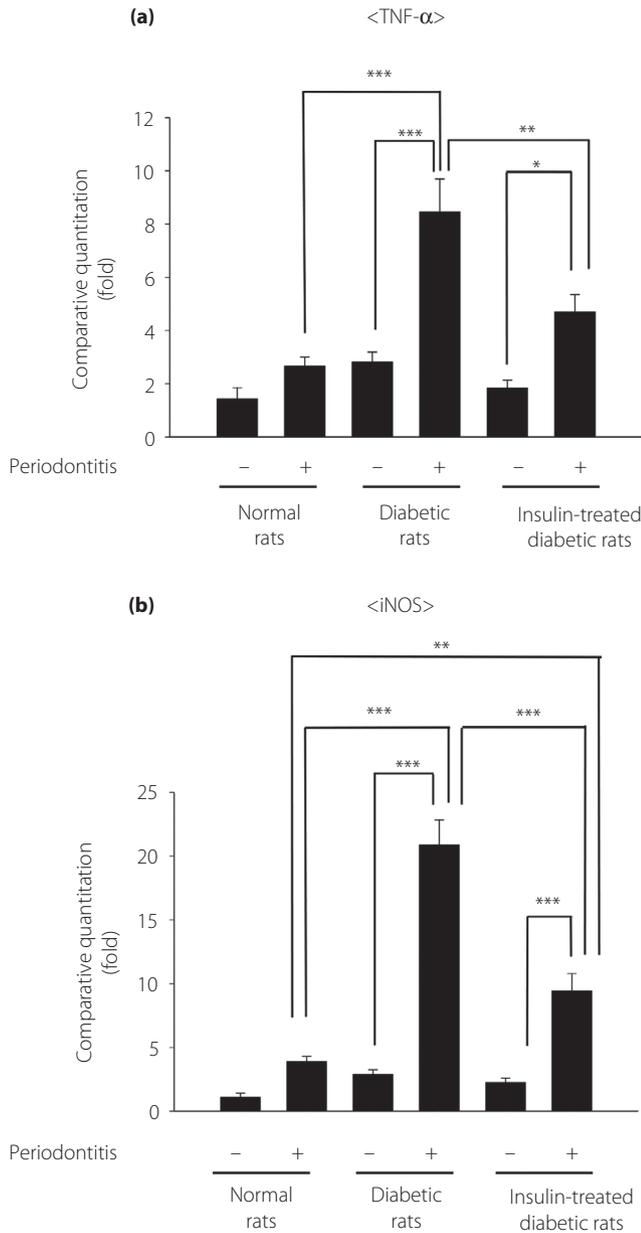*$P < 0.01$, **$P < 0.001$ vs diabetic rats. HbA1c, glycated hemoglobin.

Figure 2 | Gingival gene expression of (a) tumor necrosis factor (TNF)-α and (b) inducible nitric oxide synthase (iNOS) in the gingiva. The results are expressed as the mean ± standard error of the mean (n = 8). *P < 0.05, ** P < 0.01, ***P < 0.001.

Figure 3 | Micro-computed tomography images of the maxillae 2 weeks after the induction of periodontitis. (a) Representative micro-computed tomography image of the control and the periodontitis sides in the normal and diabetic rats. (b) Alveolar bone absorption was measured as the distance from the mesial buccal cement–enamel junction to the alveolar bone crest of the second molar. The results are expressed as the mean ± standard error of the mean (n = 6). *P < 0.05, ** P < 0.01, ***P < 0.001.

($P < 0.01$ and $P < 0.05$, respectively). Insulin significantly decreased periodontitis-induced alveolar bone resorption by 64% in diabetic rats ($P < 0.001$).

Periodontitis-induced bone resorption

The micro-CT images showed periodontitis-induced alveolar bone resorption (Figure 3a,b). The most severe bone resorption was seen on the periodontitis side of the alveolar bone of diabetic rats. Quantitative analyses showed that the bone resorption was slightly, but significantly, higher on the periodontitis side of normal rats, and on the control side of diabetic rats compared with that on the control side of normal rats
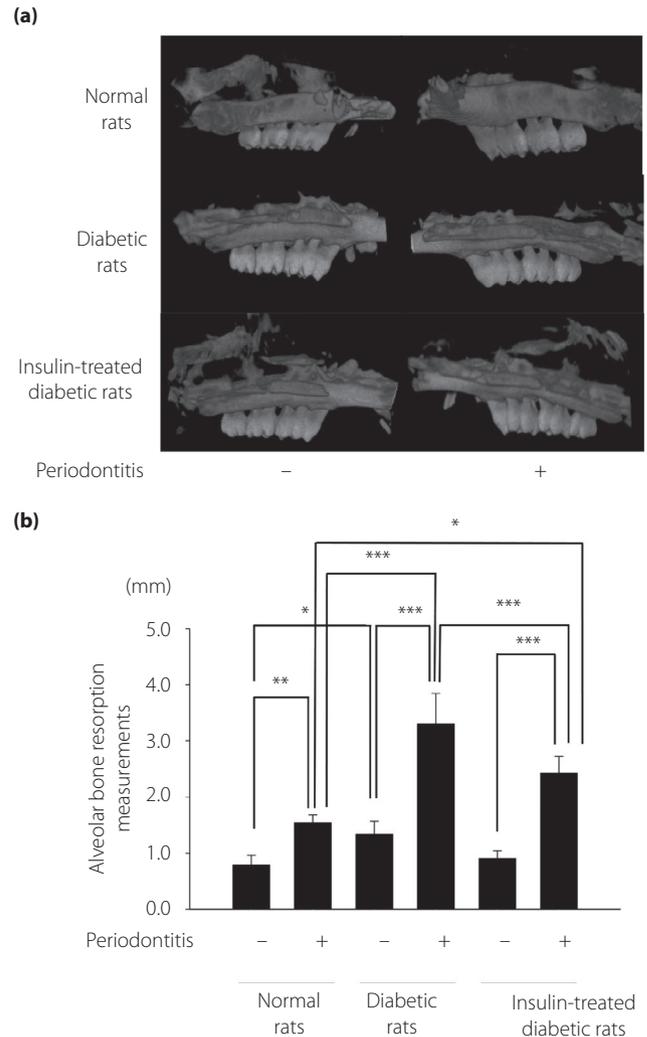
Histological evaluation of periodontal tissue

There was a slight increase in the numbers of inflammatory cells in the gingiva of the periodontitis side of normal rats and on the control side of the diabetic rats (Figure 4). In contrast, periodontitis severely increased the inflammatory cells in the gingiva of the diabetic rats. Insulin treatment decreased the inflammatory cells in the periodontitis gingiva of the diabetic rats.
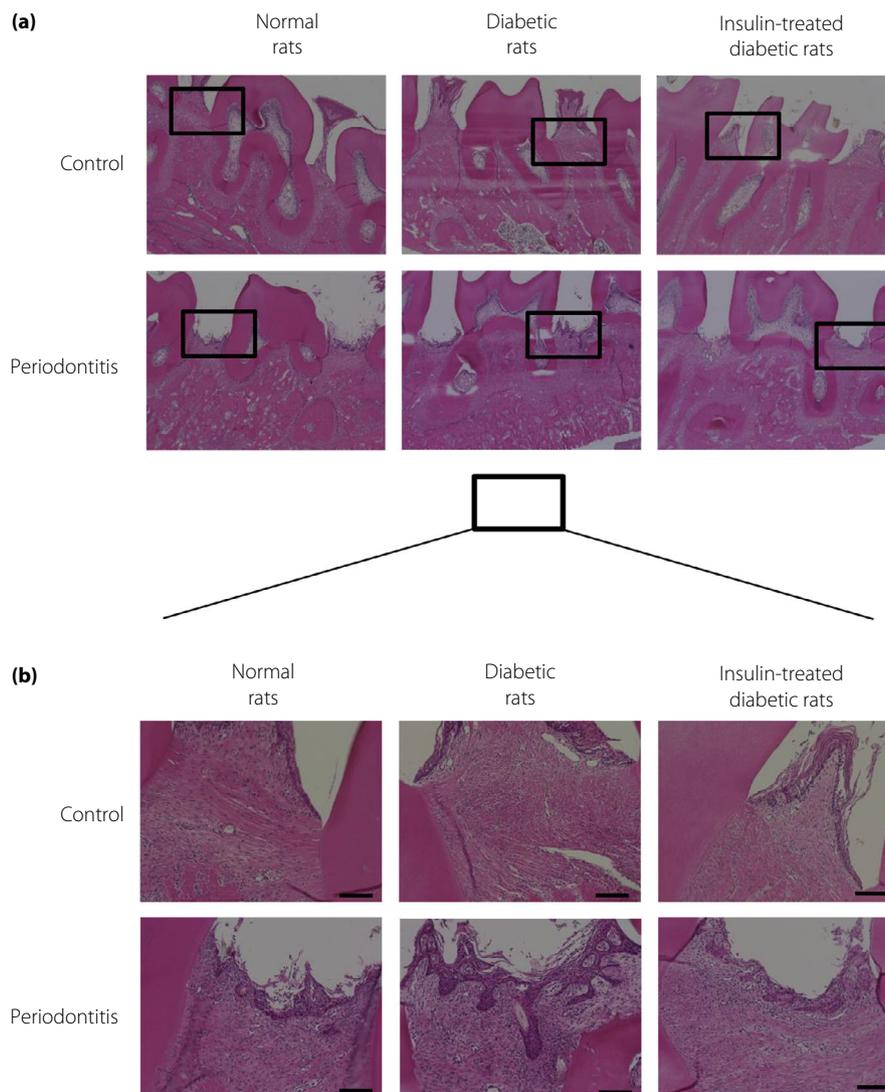
Figure 4 | (a) Representative hematoxylin–eosin staining of gingiva on the control and the periodontitis sides of normal and diabetic rats. (b) High-power representative photographs of hematoxylin–eosin staining of gingiva. Insulin treatment decreased the inflammatory cells in the periodontitis gingiva of the diabetic rats. Bar, 100 μm.

iNOS-positive cells in periodontal tissue

Periodontitis increased the number of iNOS-positive cells in the gingiva of normal rats ($P < 0.001$; Figure 5). Even on the control side of the diabetic rats, the number of iNOS-positive cells was higher than that on the control side of the normal rats ($P < 0.001$). Periodontitis induced the highest number of iNOS-positive cells in diabetic rats. Insulin treatment significantly suppressed the number of iNOS-positive cells in diabetic rats ($P < 0.001$).

Anti-inflammatory effects of insulin

To explore the direct anti-inflammatory effects of insulin, we investigated the effects of insulin on LPS-induced inflammatory cytokine production in THP-1 cells. LPS significantly increased the gene expression of the inflammatory cytokines, TNF-α and iNOS. Pretreatment with insulin significantly inhibited LPS-induced TNF-α and iNOS expression ($P < 0.001$). Insulin doses of 10 and 100 nmol/L suppressed LPS-stimulated TNF-α expression by 52.7 and 61.3%, and iNOS expression by 39.7 and 46.5%, respectively (Figure 6).

DISCUSSION

The induction of periodontitis by ligation induced severe inflammation and alveolar bone loss in STZ-induced type 1 diabetic rats. In the present study, we have provided direct evidence that insulin treatment improves periodontitis under diabetic conditions without local treatment of periodontitis.
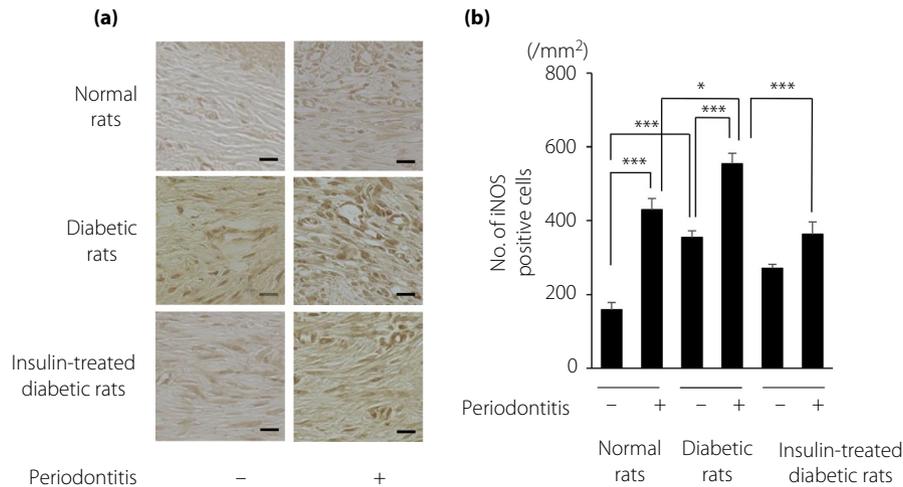
Figure 5 | (a) Immunohistological staining of inducible nitric oxide synthase (iNOS) in the gingiva. Bar, 20 μm. (b) Quantitative analyses of iNOS-positive cells in the gingiva. The results are expressed as the mean ± standard error of the mean (*n* = 6). *$P < 0.05$, *** $P < 0.001$.
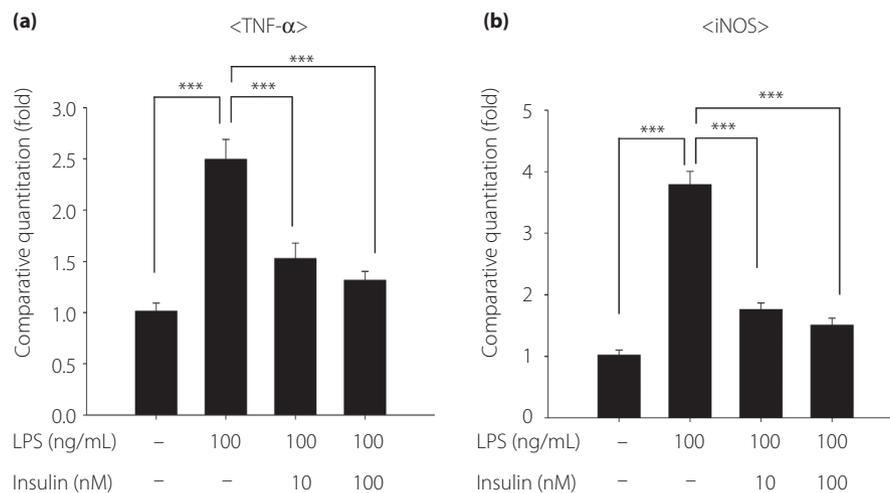

Figure 6 | Inhibitory effects of insulin on lipopolysaccharide (LPS)-induced inflammatory gene expressions. The messenger ribonucleic acid gene expression of (a) tumor necrosis factor (TNF)-α and (b) inducible nitric oxide synthase (iNOS) were determined by real-time quantitative polymerase chain reaction. Insulin was added 30 min before LPS stimulation. Then, the cells were stimulated with LPS for 4 h. Results are expressed as the mean ± standard error of the mean (*n* = 6). ***$P < 0.001$.

The ligature model of periodontitis was widely used to initiate periodontal disease in experimental animals[18–20]. Plaque accumulation around the ligature induces acute and chronic inflammation in the gingiva, subsequently leading to alveolar bone loss. The change of periodontitis occurs more acutely than human periodontitis. However, the inflammation and bone less in the ligature model are dependent on ligature accumulation of bacteria, which is similar to human periodontitis. In the present study, ligature-induced plaque accumulation induced more severe periodontitis accompanied with inflammatory cell

infiltration and increases in TNF-α and iNOS mRNA expression in diabetic rats compared with the normal condition; these findings are consistent with previous animal and clinical investigations[21–23].

In type 2 diabetes, there is a consensus that periodontitis is significantly associated with poorer glycemic control[24,25]. However, there is still controversy as to whether lowering blood glucose improves periodontal disease in type 1 diabetes patients[9,14]. In the present study, we showed that insulin treatment improved periodontitis in type 1 diabetes model rats

without any local treatment of periodontitis, suggesting the pivotal role of insulin and glycemic control on the treatment of periodontitis in type 1 diabetes.

Impairment in the microcirculation in the gingiva was observed on the control side of diabetic rats, which was consistent with our previous investigation[16]. The impairment of microcirculation was a crucial pathophysiological disorder in diabetes. Microvascular dysfunction is the common pathogenesis of diabetic microvascular complications, such as retinopathy, nephropathy and neuropathy[26–28]. The present results might suggest that the gingiva under the diabetic condition might share similar biological abnormalities and microvascular dysfunction with other diabetic complications.

In addition to gingival blood flow, several abnormalities were shown on the control side of the periodontal tissue in diabetic rats. The number of iNOS-positive cells in the gingiva and alveolar bone loss was significantly increased on the control side of the gingiva in diabetic rats compared with that in normal rats. These results might account for the high morbidity of periodontitis in the type 1 diabetes condition.

Clinical studies found a positive correlation between the gingival blood flow and the severity of gingival inflammation[29,30]. Inflammation increases and activates iNOS, which subsequently produces excess nitric oxide. In a rat model of periodontitis, the selective iNOS inhibitor, mercaptoethylguanidine, ameliorated alveolar bone loss[31]. We previously showed that iNOS gene expression and nitrotyrosine levels, a footprint of nitrosative stress, in the gingiva were increased not only by periodontitis, but also by the diabetic condition[16]. Once periodontitis was induced in the diabetic rats, iNOS gene/protein expressions and gingival blood flow were markedly increased, both of which were suppressed by insulin treatment.

An in vitro study showed that insulin had immunosuppressive effects on LPS-stimulated inflammatory cytokine expressions in THP-1 cells. Zhu et al.[32] showed that the anti-inflammatory effects of insulin were dose-dependent, but did not depend on glycemic control, by adjusting the dosage ratio of glucose and insulin in rats. There is a possibility that insulin treatment might have a direct immunosuppressive effect on periodontitis besides the glucose-lowering effect. Furthermore, Maekawa et al.[33] showed the inhibition of osteoblast differentiation and enhanced osteoclast activation with the greater alveolar bone absorption in periodontitis of STZ-induced diabetic mice. In addition, previous studies showed that insulin increased osteoblast differentiation and osteoclastogenesis[34,35]. Further study is required to show the effects beyond glucose-lowering of insulin on periodontitis in type 1 diabetes.

In summary, the gingiva in STZ-induced diabetic rats showed similar biological abnormalities and microvascular dysfunction with other diabetic complications. Insulin treatment improved basal gingival blood flow, and suppressed the periodontitis-induced inflammation of the gingiva and the alveolar bone absorption in STZ-induced diabetic rats. As the effects of insulin could be observed without local periodontal therapy,

these results suggest the pivotal role of insulin in diabetes-associated exacerbation of periodontitis in type 1 diabetes.

DISCLOSURE

The authors declare no conflict of interest.

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