Alveolar Bone Protective and Hypoglycemic Effects of Systemic Propolis Treatment in Experimental Periodontitis and Diabetes Mellitus

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ABSTRACT The aim of this study was to evaluate the efficacy of the anti-inflammatory effects of propolis on the systemic and local effects on experimental periodontitis and diabetes. Fifty-six Wistar rats were divided into seven groups: (1) negative-control (NC), (2) periodontitis (P), (3) diabetes (D), (4) diabetes+periodontitis (DP), (5) periodontitis+propolis (P-Pro), (6) diabetes+propolis (D-Pro), and (7) diabetes+periodontitis+propolis (DP-Pro). Periodontitis was induced by ligature placement and diabetes was induced by streptozotocin injection. Propolis (Pro) was administrated by oral gavage (100 mg/kg/day). On day 21, plasma was obtained for analysis and alveolar bone level was evaluated using histomorphometric analysis. Compared to NC the final blood glucose levels for D-Pro was not significantly different ($P = .052$), however, D, DP, and DP-Pro were significantly different. There were no statistically significant differences in blood glucose concentrations between P and P-Pro, between D and D-Pro, and between DP and DP-Pro. All groups showed significantly more alveolar bone loss compared with NC. A significant difference in bone loss was found between P and P-Pro, and DP and DP-Pro, however there was no difference between D and D-Pro. Plasma interleukin 1beta (IL-1 β), tumor necrosis factor-alpha (TNF- α), and matrix metalloproteinase-8 (MMP-8) levels were not significantly different among groups. In conclusion, propolis reduced fasting blood glucose levels in diabetes. In addition, propolis might be beneficial as an adjunct treatment of diabetes associated periodontitis and periodontitis without diabetes.

KEY WORDS: • diabetes mellitus • interleukin 1beta • matrix metalloproteinase-8 • periodontitis • plasma • propolis • tumor necrosis factor-alpha

INTRODUCTION

PERIODONTITIS IS ONE of the most widespread inflammatory diseases, characterized by periodontal pocket formation, clinical attachment loss, and alveolar bone destruction.¹ The major component of the soft and hard tissue destruction in periodontitis occurs as a result of the hyperactivation of the host immune-inflammatory response against pathogenic bacterial plaque.² Interleukin 1beta (IL-1 β) and tumor necrosis factor-alpha (TNF- α) induce connective tissue destruction and bone resorption by promoting differentiation of osteoclast precursors and subsequently by activating osteoclasts.^{2,3} Furthermore, IL-1 β and TNF- α are well-investigated markers of disease activity in the periodontium and they synergistically act to induce bone resorp-

tion.^{3,4} They also induce expression of other inflammatory destructive mediators like matrix metalloproteinase-8 (MMP-8).⁴ Furthermore, elevated levels of MMP-8 have been associated with severe periodontal inflammation.⁴

Diabetes mellitus is a systemic disease characterized by hyperglycemia. Type 1 diabetes results from an autoimmune-mediated destruction of insulin-producing β cells, whereas type 2 results from insulin resistance rather than from the total absence of insulin production. Diabetes mellitus is a growing global health problem leading to several complications and despite full compliance with diet and drug administration and the absence of any concurrent illness, diabetics may display poor glycemic control. In 1993, periodontal disease was considered the sixth complication of diabetes mellitus along with the five classical complications of retinopathy, nephropathy, neuropathy, macrovascular disease, and impaired wound healing.⁵

Both diabetes mellitus and periodontal disease are chronic inflammatory disorders with inflammation as a central feature of their pathogenesis.^{2,6} The interaction between individual

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inflammatory diseases may permit one to affect the incidence and severity of the other.⁷ The pathogenesis of diabetes-associated cytokine imbalance is still unclear. Moreover, the potential of periodontitis to affect diabetes has been a matter of great interest. However, there is inadequate information regarding their cytokine interactions in peripheral blood.

The adjunctive use of anti-inflammatory agents can increase predictable therapeutic response and slow the progression of inflammatory disorders like periodontitis and diabetes mellitus. Propolis is a resinous material, which contains more than 300 different compounds that honeybees collect from various plant species.⁸ Propolis has received increased attention due to its biological and pharmacological properties, which include anti-inflammatory and immunomodulatory actions,⁹ and anti-diabetic and antihyperglycemic properties.^{10,11}

This study was designed to determine the effects of propolis on ligature-induced periodontitis and/or streptozotocin (STZ)-induced diabetes on plasma levels of IL-1 β , TNF- α , and MMP-8, on alveolar bone loss, and on plasma levels of glucose.

MATERIALS AND METHODS

Experimental design

A power analysis with G*Power (version 3.1.7, Franz Faul; Christian-Albrechts-University, Kiel, Germany) was performed to estimate the sample size. It showed that a total sample size of 56 rats would give 84% power (actual power, 0.8410; critical F, 2.290432; noncentrality parameter, 16.94), to detect significant differences with a 0.55 effect size and at an $\alpha=0.05$ significance level. Male Wistar albino rats (300–350 g) were housed with food and water *ad libitum*, at a constant room temperature, under a 12-h light/dark cycle. Prior to the procedures, all animals were allowed to acclimate to the laboratory environment for 1 week. The rats were randomly divided into seven groups of eight animals each: (1) negative-control (NC); (2) periodontitis (P); (3) diabetes (D); (4) diabetes + periodontitis (DP); (5) periodontitis + propolis (P-Pro) 100 mg/kg; (6) diabetes + propolis (D-Pro) 100 mg/kg; and (7) diabetes + periodontitis + propolis (DP-Pro) 100 mg/kg.

The research protocol was approved by the Local Animal Research Ethics Committee and performed in accordance with Guiding Principles for Research Involving Animals and Human Beings, Recommendations from the Declaration of Helsinki.

Periodontitis induction

After an overnight fast, the animals were anesthetized with ketamine (Ketalar; Pfizer, New York, NY, USA) (1 mL/kg i.p.) and xylazine chloride (Rompun; Bayer, Leverkusen, Germany) (0.1 mL/kg i.p.) for subgingival placement of 4.0 silk ligatures around both upper first molars.¹² The ligatures were kept in place during the experimental period and served as a retention device for oral bacteria. The ligatures were examined daily during the administration of propolis or vehicle by oral gavage. They were replaced if they were

loose or missing. Nonperiodontitis groups received only general anesthesia. Ligatures were placed after confirmation of diabetes induction.

Diabetes induction

STZ (Sigma, St Louis, MO, USA) that was freshly dissolved in 0.1 M citrate buffer (pH 4.5) was administered by intraperitoneal injection (50 mg/kg) as a single dose to induce diabetes.¹³ Nondiabetic groups received only the citrate buffer injection. Diabetes was confirmed by analysis of blood glucose after an overnight fast, using a blood glucose meter (AccuChek; Roche, Penzberg, Germany), 48 h after STZ injection. Animals with blood glucose levels greater than 300 mg/dL were considered diabetic. Blood glucose levels were measured without anesthesia in all animals by testing blood samples (5 μ L) that were obtained through the tail vein using insulin syringes (1 mL), before the experimental period and at the end of the study. At the time of sacrifice, laboratory tests were also performed to confirm the blood glucose levels.

Propolis preparation and administration

The solid propolis was dissolved in an extractor in boiling ethanol. This extract was cooled and the wax removed by filtration. The concentration of this filtrate was accomplished in a rotary evaporator at room temperature until a thick paste was formed. By adding ethanol to this extract in a volumetric flask, a 10% concentration of propolis (Pro) was obtained. The propolis was analyzed using gas chromatography–

TABLE 1. CHEMICAL CONSTITUENTS OF PROPOLIS

RT	Constituents	%TIC
Phenolic compounds		
27.93	4,5 Dimethoxy-(2-propenyl) 2-phenol	1.25
31.06	Pinocembrin	14.75
34.12	Chrysin	7.67
34.84	Galangin	4.90
Organic and fatty acids		
9.03	Decanoic acid	0.23
13.31	4-Pentenoic acid	1.74
20.30	Cinnamic acid	0.29
20.73	3-Hydroxy-4-methoxycinnamic acid	1.82
16.74	2-Propenoic acid	2.70
20.91	3,4-Dimethoxycinnamic acid	3.40
22.93	Coumaric acid	0.19
25.12	9-Octadecanoic acid	2.05
25.49	Octadecanoic acid	0.21
Alcohol, ketones, and terpenes		
8.02	2-Propen-1-ol	0.21
34.38	5-3,3-Dimethyl-cyclohexanone	1.36
24.49	2-Nonadecanone	0.66
15.22	Gamma-eudesmol	0.37
15.66	Beta-eudesmol	0.38
15.71	Alpha-eudesmol	0.59
16.26	Alpha-bisabolol	0.17
29.72	2-Propen-1-one	15.30

RT, retention time; TIC, total ion current.

mass spectrometry to determine its components (Table 1). Animals received a daily oral gavage with 100 mg/kg of propolis for 21 days, while nonpropolis groups received vehicle alone.

Blood samples and determination of plasma cytokine and MMP levels

After 3 weeks, animals were anesthetized and ~8 mL of blood was collected from each animal via cardiac puncture (10 mL syringe with heparin) without opening the abdominal cavity. Blood samples were transferred to heparin-coated tubes (10 mL). Plasma was obtained by centrifugation (3000 g for 10 min) and immediately stored at -80°C for later examination. The animals were sacrificed using an anesthesia overdose and neck-vertebra dislocation. Plasma IL-1 β (ELISA kit; Boster, Pleasanton, CA, USA), TNF- α (ELISA kit; RayBiotech, Norcross, GA, USA), and MMP-8 (ELISA kit; RayBiotech) levels were determined using ELISA kits according to the manufacturer's instructions.

Histological analysis

The maxillae were dissected, fixed in 10% formalin solution pH 7.2 for 48 h, washed in water, and demineralized in 10% glacial acetic acid for 3 months. At the end of demineralization, each maxilla was washed in running water for 24 h, dehydrated with serial alcohol baths, and cleared. Step serial sections of 4 μm in thickness were cut in a mesiodistal direction of paraffin-embedded blocks. Sections were stained with Masson's trichrome.

The linear distance from the cemento-enamel junction to the alveolar bone crest in the interproximal areas between the first and second molars were measured,¹⁴ using a light microscope (Olympus Bx51, Tokyo, Japan) with a camera attachment (Olympus DP-71 Digital Camera) that utilized a software program (AnalySIS 2.1 Soft-Imaging Software GmbH, Münster, Germany) as shown in Figure 1. The measurements were made by an examiner who was masked to the samples (Birkan Yakan). At least 10 sections were measured for each site. Using a previously described breeding protocol all rats were maintained periodontitis-free at baseline.¹⁵

Statistical analysis

Data were expressed as mean $\pm$ standard deviation. The normal distribution of data was determined using a Kolmogorov-Smirnov test. One-way analysis of variance (ANOVA) followed by multiple comparisons using a *post hoc* Dunnett T3 test or nonparametric Median or Kruskal-Wallis tests and *post hoc* pairwise comparisons of groups were performed. Differences were considered statistically significant at $P < .05$.

RESULTS

Blood glucose

There were no statistically significant differences in mean glucose levels between the groups at baseline, as shown

in Table 2. Analysis did show a statistically significant difference between groups in final blood glucose levels on the day of sacrifice (Table 2). Compared to NC the final blood glucose levels for P, P-Pro, and D-Pro ($P = .052$) were not significantly different, however, D ($P = .002$), DP ($P < .0005$), and DP-Pro ($P = .019$) were significantly different (Fig. 2). Within nontreatment groups, final blood glucose in DP was significantly greater compared with P ($P = .001$) and to D ($P > .05$). D was significantly greater compared with P ($P = .005$). Between propolis treatment and nontreatment groups there were no statistically significant differences between P and P-Pro, D and D-Pro, and DP and DP-Pro (Fig. 2).

Alveolar bone loss

Histological analysis of the negative-control group showed that animals were periodontitis-free and there was no alveolar bone loss in the NC group. Statistical analysis showed a significant difference between groups in alveolar bone level (Table 2). Compared with NC, all groups showed significantly more bone loss (Fig. 3). Within nontreatment groups, DP showed more bone loss when compared with D ($P = .0003$) and to P ($P = .317$). P presented significantly more bone loss compared with D ($P = .00001$). When comparisons were made between treatment and nontreatment groups, alveolar bone loss in P-Pro was significantly lower than P ($P = .001$). DP showed greater bone loss compared with DP-Pro ($P = .046$). There was no statistical significance between D and D-Pro groups ($P > .05$, Fig. 3).

Plasma cytokine and MMP-8

Plasma IL-1 β , TNF- α , and MMP-8 were not different among the groups at the end of the study (Table 2).

DISCUSSION

It is evident that some pathways of experimental periodontitis differ from human chronic periodontitis progression. The experimental periodontitis model has an acute course of inflammation, which is induced by tissue trauma during placement of a ligature and adjacent bacterial accumulation leads to further destruction of periodontal tissues.¹⁶ Although the basis of conventional periodontal treatment is scaling and root planing in humans, the treatment of experimental periodontitis in rats is difficult, because the molars of rats are smaller than those of humans. So instead of performing any sort of periodontal treatment such as scaling and root planing, only a host modulation therapy was chosen in this study.

Although there are some limitations for every animal model of a human disease, rodents such as rats are commonly used for both ligature-induced periodontitis and STZ-induced diabetes models.¹⁷ Moreover, animal models are necessary to prove cause and effect relationships and to test the potential of novel therapeutics.¹⁸

Propolis (Pro) has been shown to have anti-inflammatory and immunomodulatory properties.⁹ This study evaluated

FIG. 1. Photographs illustrating alveolar bone status between the first and second molars (magnification of $\times 4$). Arrows indicate alveolar bone crest level. Bars define metric magnification. Masson's trichrome staining was used. (A) Negative-control (NC) group (B) periodontitis (P) group (C) diabetes (D) group (D) diabetes + periodontitis group (E) periodontitis + propolis (P-Pro) 100 mg/kg group (F) diabetes + propolis (D-Pro) 100 mg/kg group (G) diabetes + periodontitis + propolis 100 mg/kg group.

the effects of host modulation with anti-inflammatory propolis administration as a treatment method.

In this study, mean plasma IL- 1β levels increased in nontreatment groups and decreased in propolis treatment groups although the differences were not statistically significant. In addition, plasma TNF- α levels were similar between groups. Nishihara *et al.*¹⁹ evaluated serum TNF- α levels for 2 weeks in KKAY diabetic mice and their lean controls after *Porphyromonas gingivalis* inoculation in the scalp. Both groups showed a significant increase in serum TNF- α levels. How-

ever, a significantly greater increase was observed in diabetics. The maximum levels of TNF- α in KKAY mice were attained on the third day, after which it steadily declined. Takano *et al.*²⁰ in a similar study, showing that serum TNF- α levels of diabetic mice and controls significantly increased after *P. gingivalis* challenge on the third day. These different results may be due to differences in experimental periodontitis induction methods and examination time.

Mean plasma MMP-8 levels tended to decrease in the nontreatment groups and increase in propolis treatment

TABLE 2. GENERAL CHARACTERISTICS OF THE STUDY GROUPS

	Control group	Nontreatment groups			Treatment groups			P-value
	NC	P	D	DP	P-Pro	D-Pro	DP-Pro	
Initial blood glucose (mg/dL)	110.6±17.1	111.0±15.5	109.0±4.7	117.4±10.0	109.3±6.1	115.4±9.7	110.9±10.1	.472
Final blood glucose (mg/dL)	122.8±14.1	128.8±8.9	413.3±78.7	450.9±94.6	132.0±24.4	319.4±129.0	365.1±59.6	<.05
CEJ-ABC (mm)	0.41±0.08	1.80±0.25	0.71±0.12	2.03±0.41	1.19±0.16	0.73±0.09	1.54±0.28	<.05
IL-1 β (pg/mL)	45.3±12.8	46.4±11.0	48.7±7.3	44.4±14.6	38.7±4.2	40.5±8.1	35.2±4.1	.066
TNF- α (pg/mL)	31.2±1.3	31.8±1.1	33.7±4.1	33.6±4.3	33.0±1.4	36.3±8.9	33.2±4.9	.431
MMP-8 (pg/mL)	3.5±0.8	3.2±1.1	3.0±0.8	2.4±0.7	3.4±0.8	3.1±1.2	3.4±0.9	.274

Data are presented as mean±standard deviation.

Comparisons were made between all groups (one-way ANOVA, Kruskal–Wallis or Median Tests).

ANOVA, analysis of variance; CEJ-ABC, cemento-enamel junction to the alveolar bone crest; D, diabetes; DP, diabetes+periodontitis; DP-Pro, diabetes+periodontitis+propolis; D-Pro, diabetes+propolis; IL-1 β , interleukin 1beta; MMP-8, matrix metalloproteinase-8; NC, negative-control; P, periodontitis; P-Pro, periodontitis+propolis; TNF- α , tumor necrosis factor- α .

groups reaching levels similar to the NC group, but the changes were not significant (Table 2). Marcaccini *et al.*²¹ showed higher plasma MMP-8 levels in periodontitis patients and periodontal treatment decreased MMP-8 levels after 3 months compared with healthy controls. Recently, in addition to tissue destructive properties of MMP-8, protective and defensive anti-inflammatory properties have been reported.⁴ However, there are no studies that report the serum levels of MMP-8 in diabetic patients with periodontitis.

In this study, STZ-induced diabetes may have increased the severity of ligature-induced periodontitis as it is seen that bone loss in the DP group was higher compared with the P group ($P=.317$). Holzhausen *et al.*¹³ found similar results at all time periods for short-term diabetes using analysis of radiographic data. Pontes Andersen *et al.*²² evaluated short-term bone loss in prediabetic rats and showed that all groups, including prediabetics with periodontitis, significantly differed from lean controls. In another study, Pontes Andersen *et al.*¹² reported that long-term bone loss in type 2

diabetic Goto–Kakizaki rats was significantly greater than controls after 6 weeks of induced periodontitis.

In this study, propolis administration significantly reduced alveolar bone loss in the periodontitis group, which is in agreement with Toker *et al.*²³ who evaluated bone loss at day 11. Furthermore, propolis treatment significantly reduced bone loss in the DP group. However, there was no statistically significant difference between the D and D-Pro groups. Propolis can prevent bone loss in cell cultures with the effects of one of its active components, caffeic acid phenethyl ester (CAPE) through the suppression of cell signaling pathways of RANKL induced NF- κ B and NFAT activity.²⁴ However, additional possible pathogenic mechanisms of diabetes related bone loss have been reported. Liu *et al.*²⁵ found that diabetic animals presented with a more persistent and severe inflammatory response and had reduced bone formation. They also reported that diabetes

FIG. 2. Final blood glucose of groups. Bars express mean±standard deviation. * Comparisons were made between NC and other groups. D, DP, and DP-Pro significantly differed from NC ($P<.05$). † D was significantly greater compared with P ($P<.05$). ‡ DP was significantly greater compared with P ($P<.05$). § DP was significantly greater compared with P-Pro ($P<.05$). || DP-Pro was significantly greater compared with P-Pro ($P<.05$). Color images available online at www.liebertpub.com/jmf

FIG. 3. Cemento-enamel junction to the alveolar bone crest (CEJ-ABC) measurements of groups. Bars express mean±standard deviation. * Comparisons were made NC versus other groups. All groups significantly differed from NC ($P<.05$). † P was significantly greater compared with D ($P<.05$). ‡ DP was significantly greater compared with D ($P<.05$). § P was significantly greater compared with P-Pro ($P<.05$). || DP was significantly greater compared with DP-Pro ($P<.05$). Color images available online at www.liebertpub.com/jmf

increased apoptosis and decreased the number of osteoblasts and periodontal ligament fibroblasts. Naguib *et al.*²⁶ showed that TNF- α expression, due to cytokine dysregulation in connective tissue in diabetic mice, was prolonged following *P. gingivalis* inoculation. Graves *et al.*²⁷ indicated that macrophages and monocytes secrete more cytokines in response to periodontal pathogens in diabetics. In addition, Mishima *et al.*²⁸ found that diabetes may also affect bone turnover. In general, such mechanisms could lead to increased periodontal bone loss in the presence of diabetes. In this study, we evaluated alveolar bone loss only by histomorphometric analysis. Therefore, our study has limitations for explaining local effects of periodontitis and diabetes, and systemic treatment of propolis. Possible effects of propolis administration on these additional mechanisms of diabetes should be investigated in further studies.

In this study, final blood glucose in the DP group was not significantly greater compared with the D group. Animal studies using different diabetic models in the presence of ligature-induced periodontitis reported similar results, which indicated that ligature placement increased plasma glucose.^{12,13} In addition, *P. gingivalis* inoculation in the scalp produced elevated blood glucose levels the third and fifth days in diabetic mice.¹⁹ In this study, there were no significant differences between propolis treatment groups or between treatment and nontreatment groups. Fuliang *et al.*¹⁰ assessed the effect of propolis on blood glucose in alloxan-induced diabetic rats. They found that blood glucose levels of propolis groups were decreased compared with the diabetic control group by the third week, and the difference was statistically significant by week 8. Li *et al.*¹¹ also found that propolis reduced blood glucose levels in STZ-induced diabetes.

Diabetes-related prolonged hyperglycemia contributes to a hyperinflammatory state by inducing nonenzymatic glycation and oxidation of lipids and proteins such as collagen, resulting in the production of advanced glycation end products (AGE) and their receptors (RAGE) that interact with monocyte/macrophage receptors and increase the production cytokines such as IL-1 β and TNF- α .^{7,27} Hyperglycemia can also lead to direct cellular damage through stimulation of intracellular pathways.¹⁷ In addition, hyperglycemia induces the production of IL-1 β in human pancreatic β -cells leading to impaired insulin secretion, decreased cell proliferation, and apoptosis.²⁷ In this study, propolis reduced hyperglycemia as shown by a decrease in blood glucose in the D group compared with NC. An explanation of the effect of propolis on diabetes is that systemic propolis administration might have prevented the destruction of β -cells. In addition, another explanation is through the reduction of AGE so the reasons for the reduction of plasma IL-1 β levels needs to be clarified in future studies.

Some recent studies have investigated carriage systems of propolis micro particles for the treatment of periodontal disease.^{29,30} Subgingival irrigation with propolis extract as an adjuvant to periodontal treatment was found to be more effective than conventional treatment, both by clinical and microbiological parameters.³¹ This study showed useful effects

of propolis on alveolar bone loss in diabetes-associated periodontitis. Therefore, clinical studies evaluating the effects of propolis on periodontitis patients with diabetes mellitus should also be investigated in future studies.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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