

# Protective effects of puerarin on the periodontium in an experimental rat model of periodontitis with and without diabetes mellitus: A stereological and immunohistochemical study

Cigdem Coskun Turer<sup>1</sup>, Akif Turer<sup>2</sup>, Gamze Altun<sup>3</sup>

<sup>1</sup>Bulent Ecevit University, School of Dentistry, Department of Periodontology, Zonguldak, Turkey

<sup>2</sup>Bulent Ecevit University, School of Dentistry, Department of Oral and Maxillofacial Surgery, Zonguldak, Turkey

<sup>3</sup>ndokuz Mayıs University, School of Medicine, Department of Histology and Embryology, Samsun, Turkey

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## Abstract

**Aim:** Periodontitis is a chronic inflammatory disease characterized by tissue destruction. Patients with diabetes mellitus are more susceptible to severe tissue destruction. Puerarin, a biological component derived from *Pueraria lobate*, has anti-inflammatory and anti-diabetic effects. The purpose of the study is to evaluate the protective role of puerarin on alveolar bone loss and connective tissue destruction in ligature induced diabetic and non-diabetic rats.

**Material and methods:** Sixty rats were divided as non-diabetic control, non-diabetic-experimental periodontitis (EP)-systemic saline, non-diabetic-EP-systemic puerarin, diabetic control, diabetic-EP-systemic saline and diabetic-EP-systemic puerarin. Diabetes was induced by injection of streptozocin (200 mg/kg). EP was achieved by placing a sterile silk suture around the first molars into the gingival sulcus for 15 days. In puerarin treated groups, 200 mg/kg puerarin was applied daily for 15 days beginning one-day prior the placement of the ligature. The alveolar bone level of the first molar tooth, alveolar bone ratio in the furcation area and the attachment level were evaluated histologically. MMP-9, TIMP-1 levels and RANKL/OPG ratio were evaluated immunohistochemically.

**Results:** Significant reduced alveolar bone and attachment losses were found in puerarin-treated groups comparing to in saline-administered groups ( $p < 0.05$ ). There was a significant reduction of RANKL/OPG ratio and MMP-9 levels and an increase in TIMP-1 levels in puerarin-treated groups compared with saline-administered groups during the induction of experimental periodontitis in both diabetic and non-diabetic condition ( $p < 0.05$ ).

**Conclusion:** Puerarin might help to prevent alveolar bone loss and connective tissue destruction in periodontal disease in diabetic and non-diabetic condition.

**Keywords:** Immunohistochemistry; Periodontitis; Rats; Streptozocin Diabetes.

## INTRODUCTION

Periodontitis is a chronic disease that leads to the destruction of tooth-supporting tissues (1). Destructive lesions result from the host immune/inflammatory response to subgingival biofilm (2). Cytokines and matrix metalloproteinases (MMPs) are involved in the process (2). Proteases derived from bacteria and the host are known to play important roles in the destruction process. The periodontium consists of various extracellular matrix components in addition to fibrous components (3). MMPs are responsible for the remodeling and destruction of the matrix components and collagen (4). MMP activity is normally controlled by endogenous inhibitors, called tissue inhibitors of metalloproteinases (TIMPs) (3,5). The balance between MMP and TIMP levels plays an important

role in the disease process. Increased MMP and decreased TIMP levels are responsible for the initiation of collagen degradation in connective tissue and alveolar bone (6).

MMP-9, a member of the gelatinase family, is synthesized mainly by polymorphonuclear leukocytes (PMNLs) and degrades the type IV collagen found in gingival tissues (6). The main inhibitor, TIMP-1, is a glycoprotein of 30 kDa that can be synthesized by many cell types (4). In the disease process, bone destruction accompanied by connective tissue attachment damage occurs; both are carried out by osteoclasts. Various mediators, such as interleukin-1 beta (IL-1 $\beta$ ), prostaglandin E2 (PGE2), and tumor necrosis factor-alpha (TNF- $\alpha$ ), can act as activators of osteoclasts (7). Another important system that enables the activation of osteoclasts includes receptor activator of

Received: 02.01.2018 Accepted: 29.01.2018 Available online: 08.02.2018

Corresponding Author: Cigdem Coskun Turer, Bulent Ecevit University, School of Dentistry, Department of Periodontology, Zonguldak, Turkey, E-mail: cigdemturer@gmail.com

nuclear factor kappa-B (RANK), RANK ligand (RANKL), and osteoprotegerin (OPG). RANK is a receptor expressed by osteoclast progenitor cells. RANKL and OPG are cytokines belonging to the TNF family, produced by osteoblasts and bone marrow stromal cells (8). RANKL stimulates osteoclast activation, and OPG has the opposite role. RANKL and RANK create active osteoclasts from progenitor cells, whereas the OPG-RANKL combination inhibits the differentiation process. Thus, an increased RANKL/OPG ratio indicates the occurrence of the destruction process (9).

Several modifying factors have profound effects on the host and make the individual more susceptible to periodontal disease (10). Diabetes mellitus (DM), one modifying factor, is a complex disease caused by impaired production or deficient utilization of insulin. It has effects on the vascular system, immune system, inflammatory response, physiological responses, and tissue repair (11). Thus, DM has the potential to modify susceptibility to disease, the plaque microbiota, the clinical presentation of periodontitis, disease progression, and the response to treatment (11).

The degree of oral and systemic complication depends on the extent of glycemic control in DM (12). Inflammation is a major feature of diabetes and periodontal disease. In diabetic patients, inflammatory courses are upregulated in periodontal tissues. DM with poor glycemic control leads to an increased risk of periodontal tissue destruction and causes a more severe course of periodontitis (13).

Puerarin is a major bioactive component and traditional Chinese medicine obtained from the root of *Pueraria lobate* (14). It is used widely in the treatment of cardiovascular diseases, cerebrovascular disorders, cancer, Parkinson's disease, Alzheimer's disease, and diabetes (15). It also has protective effects against inflammation (15).

Puerarin has been used in DM treatment since 1990 in China (15). Its benefits in the treatment of diabetes depend on the ability to reduce insulin resistance; it also reduces blood glucose levels when administered intravenously (16). Moreover, puerarin has a protective effect against oxidative stress in pancreatic islet cells (17). Thus, puerarin has an important role in protecting islet cells and it may be considered as a candidate drug for DM (15).

Puerarin also has anti-inflammatory properties. It suppresses the expression of C-reactive protein, inducible nitric oxide synthase, and cyclooxygenase-2, and inhibits the nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway (18). Puerarin lowered the levels of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-8, PGE2, and nitric oxide and inhibited the expression of MMP-2 and MMP-9 (19,20). It also increased the expression of OPG and prevented RANKL expression (21).

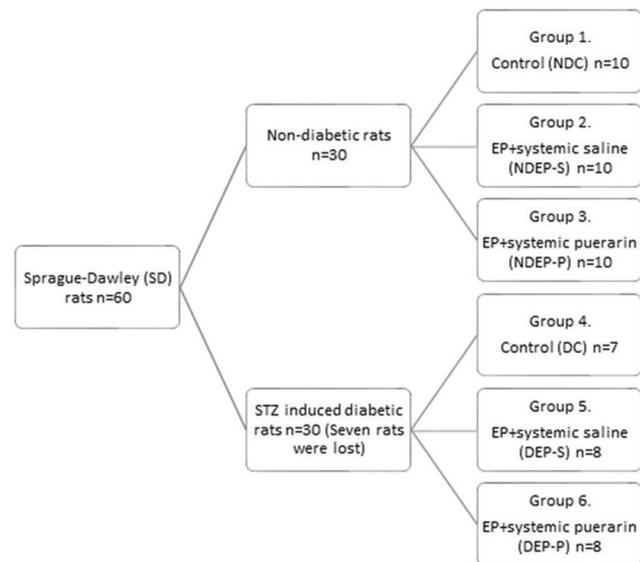
Given this background, we hypothesized that puerarin would effectively suppress periodontal inflammation and alveolar bone destruction. The purpose of this study was to investigate the effects of systemically administered

puerarin on alveolar bone destruction and collagen degradation by evaluating changes in the RANKL/OPG ratio and MMP-9 and TIMP-1 levels in a ligature-induced periodontitis model in diabetic and non-diabetic rats.

## MATERIAL and METHODS

### Animals and experimental design

The research protocol was approved by the Bulent Ecevit University Animal Experiments Committee (no. 2015-12-03/06). In total, 60 male Sprague Dawley rats, weighing 250–300 g, were used. Body weights were measured weekly to ensure adequate feeding before and after surgery. All rats were housed separately in plastic cages. They were kept in a temperature-controlled room with a standard light/dark illumination cycle (12/12 h), and maintained on food and water ad libitum. The rats were divided into six groups: group 1 (n = 10), non-diabetic control (NDC); group 2 (n = 10), non-diabetic–experimental periodontitis (EP)–systemic saline (NDEP-S); group 3 (n = 10), non-diabetic–EP–systemic puerarin (NDEP-P); group 4 (n = 7), diabetic control (DC); group 5 (n = 8), diabetic–EP–systemic saline (DEP-S); and group 6 (n = 8), diabetic–EP–systemic puerarin (DEP-P; Figure 1).



**Figure 1.** Design of experimental groups. EP: Experimental periodontitis (ligature kept 15 for days); STZ: Streptozotocin.

### Preparation and administration of puerarin

Puerarin was prepared by dissolving it in 0.5% carboxymethylcellulose on the day of use. Puerarin (200 mg/kg) was administered once daily by gavage for 15 days starting 1 day prior to ligature placement in DEP-P and NDEP-P groups. The DEP-S and NDEP-S groups received saline once daily by gavage for 15 days beginning 1 day prior to ligature placement.

### Surgical procedure

Induction of diabetes mellitus: Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) solution (200 mg/kg). Rats were fasted for 16 h prior to injection. Freshly prepared 0.1-M citrate buffer was added

to STZ powder (Sigma 85882) to a final concentration of 20 mg/mL. On the first day of injection, the rats were fed with 10% sucrose. The next day, rats were fed with fresh water only. Body weight and blood glucose level after injection were measured at least once daily and more frequently when necessary.

**Induction of periodontitis:** All surgical procedures were performed under anesthesia with intramuscular injection of 3 mg/kg xylazine hydrochloride (Rompun; Bayer, Leverkusen, Germany) and 35 mg/kg ketamine hydrochloride (10% Ketasol; Richter Pharma AG, Wels, Austria). For each animal in the NDEP-S, NDEP-P, DEP-S, and DEP-P groups, after aseptic preparation, a sterile 3.0 silk ligature was placed around the mandibular right first molar into the gingival sulcus; to allow for microbial dental plaque deposition and inflammation, the ligature was kept in place for 15 days. The experimental period ended with the euthanization of the rats by general anesthesia overdose.

#### **Histological and immunohistochemical analysis**

Following the experimental period, the mandibular bone was removed; sections were fixed in 10% buffered formalin for 10 days. The specimens were decalcified in 10% formic acid (Merck Millipore Corporation; Darmstadt, Germany) at room temperature (pH 7.2) for 4 weeks and after routine tissue processing, they were embedded in the paraffin (Agar, Cambridge, UK).

Serial paraffin sections were taken mesiodistally along the mandibular first molars. Sections of 10  $\mu$ m were obtained using a rotary microtome (Leica RM 2135; Leica Instruments, Nussloch, Germany) for light microscopic examination and stereological analyses considering the systematically unbiased random sampling strategies. According this, each 20th section was selected.

The alveolar bone level (ABL) of the first molar tooth, alveolar bone area (ABA) and the attachment level (AL) were evaluated in all sections. The ABL of the first molar tooth were measured from the cemento-enamel junction (CEJ) to alveolar bone crest (ABC). The sum of the trabecular bone area and the bone marrow area were considered as the ABA. AL was determined by calculating the area between the CEJ and the most coronal portion of connective tissue attachment (CTA) to cementum. All analysis was performed by a calibrated examiner who is blinded to the study design with respect.

In the immunohistochemical analyses, a streptavidin-biotin complex (Abcam, Cambridge, MA, USA) was used. The polyclonal anti-RANKL, anti-OPG, anti-TIMP-1, anti-MMP-9 (Boster Biological Technology Ltd., Fremont, USA) were used for detection. Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide for 10 min at 25°C. The slides were incubated with the primary antibody in a humid chamber overnight at 4°C. Consequently, the slides were washed with 1% PBS/BSA and incubated with biotinylated secondary antibodies (Abcam, Cambridge, MA, USA) for 60 min at room temperature. HRP/AEC chromogen kit (Abcam, Cambridge,

MA, USA) was used as chromogen and the slides were counterstained with Mayer's hematoxylin (Sigma, Saint Louis, USA). Positive cells were dyed as brown. The immunoreactivity was scored by one researcher using HScore (22). Immunohistochemical intensity was scored as 0 (absent), 1 (weak), 2 (moderate), 3 (intense). The calculation was made with this formula: HSCORE =  $\sum Pi / (i + 1)$ . According to the formula, *i* shows the intensity scores, *Pi* is the percentage of stained cells, and 1 is the correction factor.

#### **Stereological analysis**

The serial sections used for application of Cavalieri method to estimate the volume of periodontal ligament and the total volume of alveolar bone. In the Cavalieri method, the point density is designed to hit a minimum of 800 points per region of interest in estimation of all volumetric values according to pilot study. Also point counting grid was used as 250  $\mu$ m<sup>2</sup> for estimation of the volume of periodontal ligament and the total volume of alveolar bone. The appropriate coefficient of error (CE) and coefficient of variation (CV) were estimated according to Gundersen and Jensen (23).

Point Counting grids were placed on a PC screen; the number of hit points of the grid was counted for all areas of interest. The volumes in each section were estimated from the following equation: Volume (*V*):  $t \times a/p \times (\sum P)$ , where *V* is the volume of interest in one section plane, *t* is the section thickness, *a/p* is the interpoint area, and  $\sum P$  is the number of points hitting the object of interest in that section (24).

#### **Statistical analysis**

Statistical analyses were performed using the SPSS software (ver. 19.0). For normally distributed data, one-way analysis of variance (with Tukey's post hoc test) was used to compare volumetric values between groups. For non-normally distributed data, the non-parametric Kruskal-Wallis test was used. *P* values < 0.05 were considered to indicate statistical significance. Values are shown as means  $\pm$  standard deviations for each group. A posteriori power calculation yielded a power of 97% to detect differences in outcomes between groups.

## **RESULTS**

In total, seven rats died from the STZ injection in the period of DM induction. No other death occurred during the rest of the experiment. Weight loss that did not affect vital functions was observed. Blood glucose levels were measured using the tail vein and recorded with a blood glucometer and test strips (ACCU-CHEK Active; Roche Diagnostics, Mannheim, Germany). Rats with fasting glucose levels  $\geq$  16 mM were considered to be diabetic.

#### **Protective effect of puerarin on alveolar bone and attachment loss**

Table 1 shows the alveolar bone loss (ABL), alveolar bone area (ABA), and attachment loss (AL) results. There was a significantly difference between the NDC and DC groups for ABL and AL levels (*p*<0.05) also, a significantly

difference was observed in the non-diabetic and diabetic EP groups (NDEP-S, NDEP-P, DEP-S, DEP-P) compared with the non-ligated control groups (NDC, DC;  $p < 0.05$ ) for ABL and AL. When diabetic and non-diabetic groups were evaluated separately, ABA was significantly greater in puerarin-treated groups (NDEP-P, DEP-P) than in saline-administered groups (NDEP-S, DEP-S;  $p < 0.05$ ). Similarly,

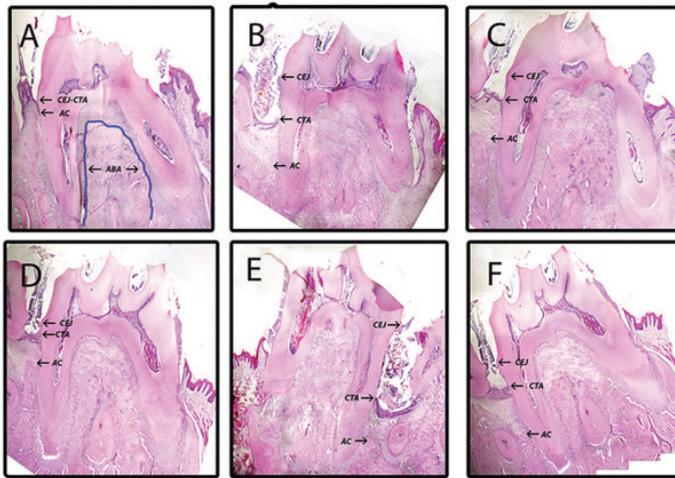
AL and ABL were significantly decreased in puerarin-treated groups (NDEP-P, DEP-P) compared with saline-administered groups (NDEP-S, DEP-S;  $p < 0.05$ ).

No significant difference in ABA, ABL, or AL was observed between the NDEP-S and DEP-P groups ( $p < 0.05$ ). Histological results are shown in Figure 2.

**Table 1. Alveolar bone area, alveolar bone level, and the attachment level among groups**

GROUP	ABA (%)	ABL (µm)	AL (µm)
Group 1.NDC (n=10)	72.19±3.39	658.05±13.78	323.03±38.06
Group 2.NDEP-S (n=10)	48.10±6.73 <sup>a,c,d</sup>	1348.05±220.31 <sup>a,c,d</sup>	1036.05±46.09 <sup>a,c,d</sup>
Group 3.NDEP-P (n=10)	59.15±4.01 <sup>a</sup>	964.04±88.82 <sup>a</sup>	664.40±16.9 <sup>a</sup>
Group 4.DC (n=7)	65.51±2.43	827.04±12.66	454.99±18.84
Group 5.DEP-S (n=8)	37.07±5.69 <sup>b,e</sup>	1668.05±69.02 <sup>b,e</sup>	1218.05±88.65 <sup>b,e</sup>
Group 6.DEP-P (n=8)	52.52±4.42 <sup>b</sup>	1248.05±62.71 <sup>b</sup>	966.49±74.20 <sup>b</sup>

Data are expressed as the mean ± SD



**Figure 2.** Histologic presentation of groups. Sections from the mesio-distal aspects throughout the mandibular first molars and the reference areas for histomorphometric analysis (H&E, 4×). (A) Group 1. NDC, (B) Group 2. NDEP-S, (C) Group 3. NDEP-P, (D) Group 4. DC, (E) Group 5. DEP-S, (F) Group 6. DEP-P. NDC, non-diabetic control; NDEP-S, non-diabetic-EP-systemic saline; NDEP-P, non-diabetic-EP-systemic puerarin; DC, diabetic control; DEP-S, diabetic-EP-systemic saline; DEP-P, diabetic-EP-systemic puerarin; CEJ, cemento-enamel junction; CTA, connective tissue attachment; AC, alveolar crest; ABA, alveolar bone area.

**Puerarin decreased the MMP-9 level and increased the TIMP-1 level**

MMP-9 and TIMP-1 immunoreactivity was assessed in the inflammatory cell infiltrate. Table 2 shows the levels of MMP-9 and TIMP-1 at the end of the experimental period. MMP-9 immunoreactivity was significantly reduced in the NDEP groups compared with the DEP groups ( $p < 0.05$ ). The puerarin-treated groups also showed significantly

less MMP-9 immunoreactivity than did the saline-treated EP groups in the diabetic and non-diabetic conditions ( $p < 0.05$ ). In contrast, TIMP-1 immunoreactivity was significantly greater in the NDEP groups than in the DEP groups ( $p < 0.05$ ). Moreover, the results indicated significantly greater TIMP-1 immunoreactivity in the puerarin-treated groups than in the saline-administered EP groups in the diabetic and non-diabetic conditions ( $p < 0.05$ ).

**Puerarin decreased the RANKL/OPG ratio**

RANKL and OPG immunoreactivity was assessed in the inflammatory cell infiltrate. Table 2 shows the levels of RANKL and OPG at the end of the experimental period. The number of RANKL-positive cells was significantly smaller in the NDEP groups than in the DEP groups ( $p < 0.05$ ). This number was also significantly smaller in puerarin-treated groups than in saline-administered EP groups in the diabetic and non-diabetic conditions ( $p < 0.05$ ). In contrast, the number of OPG-positive cells was significantly larger in the NDEP groups than in the DEP groups ( $p < 0.05$ ). OPG immunoreactivity was significantly greater in puerarin-treated groups than in saline-administered EP groups in the diabetic and non-diabetic conditions ( $p < 0.05$ ). No significant difference was found between the NDC and NDEP-P, NDEP-P and DC, NDEP-P and DEP-P, and DEP-P and DC groups (all  $p < 0.05$ ).

The RANKL/OPG ratio was significantly higher in the EP groups than in the control group under non-diabetic conditions ( $p < 0.05$ ). In addition, puerarin treatment significantly decreased the RANKL/OPG ratio ( $p < 0.05$ ). In the diabetic rat model, the RANKL/OPG ratio was significantly higher in the serum-administered EP group ( $p < 0.05$ ). No significant difference was found between the puerarin-treated and control groups ( $p < 0.05$ ).

### Stereological findings

Table 3 shows the volumes of alveolar bone and periodontal ligament. Significant difference was found between control and experimental periodontitis groups (DC-DEP-S; NDC-NDEP-S) according to the mean bone and periodontal ligament volumes both in diabetic and non-diabetic condition ( $p < 0.05$ ). Mean bone and periodontal ligament volumes were reduced significantly in the DEP

groups compared with the NDEP groups ( $p < 0.05$ ). When the diabetic and non-diabetic groups were evaluated separately, alveolar bone and periodontal ligament volumes were significantly greater in puerarin-treated groups (NDEP-P, DEP-P) than in saline-administered groups (NDEP-S, DEP-S;  $p < 0.05$ ). No significant difference in the mean bone or periodontal ligament volume was found between the NDEP-S and DEP-P groups ( $p < 0.05$ ).

**Table 2. HSCORE values of MMP-9, TIMP-1, OPG and RANKL immunoreactivity among groups**

GROUP	MMP-9	TIMP-1	OPG	RANKL	RANKL / OPG
Group 1. NDC (n=10)	103.37±3.50	250.08±5.51	249.08±6.26	113.97±5.92	0.46±0.03 <sup>f</sup>
Group 2. NDEP-S (n=10)	208.92±3.91 <sup>a,c</sup>	134.13±6.02 <sup>a,c</sup>	151.13±10.90 <sup>a,c</sup>	233.42±11.57 <sup>a,c</sup>	1.55±0.16 <sup>a,c</sup>
Group 3. NDEP-P (n=10)	129.11±4.95 <sup>a</sup>	236.62±8.47 <sup>a,f</sup>	239.47±5.33 <sup>a,f,g</sup>	162.39±3.99 <sup>a,g</sup>	0.68±0.02 <sup>a,g</sup>
Group 4. DC (n=7)	119.45±5.94	230.51±15.66	236.91±6.08	130.15±6.36	0.55±0.02
Group 5. DEP-S (n=8)	250.67±3.01 <sup>b,d</sup>	120.78±5.69 <sup>b,d</sup>	136.63±11.95 <sup>b,d</sup>	259.66±4.15 <sup>b,d</sup>	1.91±0.17 <sup>b,d</sup>
Group 6. DEP-P (n=8)	160.69±5.31 <sup>b</sup>	218.66±5.03 <sup>b</sup>	234.90±5.76 <sup>b</sup>	157.82±6.18 <sup>b</sup>	0.67±0.04 <sup>f</sup>

Data are expressed as the mean ± SD

NDC: non-diabetic control, NDEP-S: non-diabetic-EP-systemic saline, NDEP-P: non-diabetic-EP-systemic puerarin, DC: diabetic control, DEP-S: diabetic-EP-systemic saline, DEP-P: diabetic-EP-systemic puerarin.

<sup>a</sup>Significantly different from group NDC ( $p < 0.05$ ), <sup>b</sup>Significantly different from group DC ( $p < 0.05$ ), <sup>c</sup>Significantly different from group NDEP-P ( $p < 0.05$ ), <sup>d</sup>Significantly different from group DEP-P ( $p < 0.05$ ), <sup>e</sup>No significant difference from group NDC ( $p < 0.05$ ), <sup>f</sup>No significant difference from group DC ( $p < 0.05$ ), <sup>g</sup>No significant difference from group DEP-P ( $p < 0.05$ )

**Table 3. Alveolar bone volume and periodontal ligament volume among groups**

GROUP	Bone Volume mm <sup>3</sup>	PL Volume mm <sup>3</sup>
Group 1. NDC (n=10)	4.63±0.22	4.03±0.11
Group 2. NDEP-S (n=10)	3.31±0.06 <sup>b,c,f</sup>	3.01±0.13 <sup>b,c,f</sup>
Group 3. NDEP-P (n=10)	3.61±0.06 <sup>d</sup>	3.32±0.11 <sup>d</sup>
Group 4. DC (n=7)	3.87±0.06	3.50±0.14
Group 5. DEP-S (n=8)	2.42±0.13 <sup>a</sup>	1.96±0.21 <sup>a</sup>
Group 6. DEP-P (n=8)	3.45±0.04 <sup>e</sup>	3.05±0.06 <sup>e</sup>

Data are expressed as the mean ± SD

NDC: non-diabetic control, NDEP-S: non-diabetic-EP-systemic saline, NDEP-P: non-diabetic-EP-systemic puerarin, DC: diabetic control, DEP-S: diabetic-EP-systemic saline, DEP-P: diabetic-EP-systemic puerarin, PL: Periodontal ligament.

<sup>a</sup>Significantly different from group DC ( $p < 0.05$ ), <sup>b</sup>Significantly different from group NDC ( $p < 0.05$ ), <sup>c</sup>Significantly different from group DEP-S ( $p < 0.05$ ), <sup>d</sup>Significantly different from group NDEP-S ( $p < 0.05$ ), <sup>e</sup>Significantly different from group DEP-S ( $p < 0.05$ ), <sup>f</sup>No significant difference from group DEP-P ( $p < 0.05$ )

### DISCUSSION

In the present study, the protective effects of puerarin against EP were evaluated in diabetic and non-diabetic rat models. To develop a diabetic rat model, STZ was used. STZ has toxic effects on the insulin-producing pancreatic  $\beta$ -cells, leading to reduced insulin synthesis and secretion (25). The STZ model is accepted as an easy method for DM induction in many rodents (25). Deposition of advanced glycation end products, resulting from hyperglycemia, may increase inflammation. The increased inflammation in STZ-induced rats may exacerbate alveolar bone destruction (26). The diabetic rat model was selected because of the previously described effects of diabetes in accelerating the development of periodontitis. DM is associated with elevated levels of systemic markers of inflammation and increased inflammation in periodontal

tissues (27,13). Puerarin has been shown to dose-dependently lower the blood glucose level and to have positive effects in healing diabetic conditions (16,28,29). Considering the curative effect of puerarin on DM, we hypothesized that the pathogenic impact of DM could be reduced using puerarin.

In a previous study, daily intraperitoneal administration of 100 mg/kg and 200 mg/kg puerarin effectively inhibited body weight gain and ameliorated glucose and insulin intolerance (30). The safe dose of orally administered puerarin was evaluated in a rat study (31). With consideration of other studies that assessed the anti-inflammatory and anti-diabetic effects of puerarin, we determined that 200 mg/kg was suitable for use in our study (30,31).

Animal models can provide valuable data on the pathogenesis of periodontal disease (32). Previous studies have shown that plaque accumulation, ulceration of the sulcular epithelium, and growth and invasion of connective tissue are facilitated by ligature placement around teeth; eventually, loss of periodontal tissues occurs (32). The rat ligature method is easy to use in experiments (33). Another advantage of this model may be the ability to establish various systemic disease models (32). Due to the properties of the rat ligature model, a ligature-induced EP model was used in this study to create periodontitis. A disadvantage of this method is the limited duration of the ligature's effect. It is shown that ligature-induced bone loss was particularly evident on day 15 (34). Thus, the experimental period was ended on day 15 in the present study.

Cytokines and other mediators are believed to be responsible for tissue destruction. MMP-9 is one of the MMPs associated with periodontal tissue destruction (6). Because MMP activity is normally balanced with TIMPs, an imbalance between MMP and TIMP levels

plays an important role in disease progression. Reduced TIMP levels and increased MMP levels trigger collagen degradation in connective tissue and alveolar bone (6). Wide ranges of RANKL and OPG levels in gingival crevicular fluid have been documented in periodontitis and healthy conditions. However, the RANKL/OPG ratio is consistent. An increase in the RANKL/OPG ratio indicates a disease state (8). The RANKL/RANK/OPG pathway has a potential role in DM (12). Thus, the levels of MMP-9 and TIMP-1 and the RANKL/OPG ratio were estimated immunohistochemically.

Previous studies showed that puerarin might have beneficial effects and that its use is a potential therapeutic strategy for bone defects, inflammatory diseases, osteoporotic diseases, and lipopolysaccharide (LPS)-induced bone-destroying diseases (35-39). In a previous study, we found that puerarin had a positive effect on new bone formation in autogenous grafted critical-sized bone defects, with increased bone volumes in puerarin-treated groups (35). An in-vitro study in which human osteoblastic MG-63 cells were used showed that puerarin could reduce bone resorption with increased OPG secretion and decreased RANKL and IL-6 production (36). Yang et al. indicated that puerarin treatment reduced ABL and collagen destruction in rats with ligature-induced periodontitis by inhibiting the production of RANKL, IL-1 $\beta$ , TNF- $\alpha$ , MMP-2, and MMP-9 (37). Another in vitro study showed that puerarin enhanced the expression of OPG mRNA and reduced the expression of RANKL mRNA. According to these results, authors reported that puerarin might prevent bone loss in an osteoporotic mouse model, with anti-osteoporotic activity affecting the formation of osteoclasts and the expression of RANKL and OPG in osteoblasts (38).

Consistent with these studies, we found that puerarin administration increased OPG production and decreased RANKL expression in diabetic and non-diabetic rats with ligature-induced periodontitis. When the diabetic and non-diabetic groups were evaluated separately, the OPG level in puerarin-treated rats seemed to approach that in the control group with no EP. This result supports the effect of puerarin in preventing bone destruction. The similarity of the OPG levels in the diabetic and non-diabetic groups given puerarin suggests that puerarin regulates the diabetic state in addition to affecting bone metabolism. The significant difference in the RANKL level between the control and EP groups in diabetic and non-diabetic rats suggests that the RANKL level is an important mediator of bone destruction. The lack of a significant difference between the puerarin-administered diabetic and non-diabetic groups may indicate a positive effect of puerarin on the diabetic condition. The results of the present study indicate that the RANKL/OPG ratio increased with periodontal inflammation and decreased with puerarin treatment. The similarity of the RANKL/OPG ratios between the control and puerarin-treated groups shows the positive effect of puerarin on bone metabolism in the diabetic condition. As expected, MMP-9 levels were

significantly higher in the EP groups than in the control groups, in diabetic and non-diabetic rats. Similarly, the decreased TIMP-1 levels in the EP groups may support increased periodontal tissue degradation. In addition, the significant reduction of MMP-9 levels and elevation of TIMP-1 levels in puerarin-treated groups suggest that puerarin reduces tissue destruction.

Stereological examinations may be more advantageous than traditional histological examinations because they enable the volumetric (three-dimensional) examination of objects (39). Thus, in the present study, alveolar bone and periodontal ligament volumes were evaluated using stereological examinations. These volumes were lesser in EP-induced rats than in control rats, suggesting that the 15-day ligature application was sufficient to establish the EP model. In addition, the bone and periodontal ligament volumes were greater in the puerarin-treated groups than in the serum-treated groups, suggesting that puerarin alleviates the process of tissue destruction.

Our results also show that puerarin reduced ABL during the experimental period and played a role as a preventative agent in the formation of the EP model. According to our ABL, ABA, and AL results, the use of puerarin in non-diabetic rats prevented periodontal tissue destruction. It also contributed positively to periodontal status in diabetic rats. The similarity of ABA, ABL, and AL levels in the DEP-P and NDEP-S groups suggests that puerarin administration has positive effects on the diabetic condition. Thus, systematic use of puerarin may reduce periodontal destruction in the diabetic condition. Given its beneficial effects, puerarin was proposed as a candidate drug for the prevention and treatment of bacteria-induced bone-destroying diseases in a recent study.

The authors indicated that puerarin inhibited LPS-induced osteoclast formation and suppressed the release of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$ , and PGE2. They also reported that puerarin could prevent LPS-induced bone loss in vivo, and that puerarin may downregulate MMP-9 mRNA expression in a dose-dependent manner (40). Similarly, our results indicate that puerarin administration reduced the levels of pro-inflammatory cytokines and MMP-9, and increased TIMP levels, explaining the reduced collagen degradation in connective tissue and alveolar bone.

## CONCLUSION

The combined consideration of histological, immunohistochemical, and stereological results suggests that puerarin can help prevent ABL and AL in periodontal disease under diabetic and non-diabetic conditions. These findings have clinical significance, as puerarin use may prevent severe periodontal destruction in patients with DM. Although we know that the doses used in this study were not harmful to rats, we do not know the safe puerarin dose for use in humans or whether we used the most appropriate dose. Thus, the extension of our results with further studies investigating various other proteins and mediators associated with the pathogenesis

of periodontal disease is important. In addition, studies should be conducted to determine the optimal dose of puerarin.

*Competing interests: The authors declare that they have no competing interest.*

*Funding: There are no financial supports*

*Ethical approval: The research protocol was approved by the Animal Experiments Committee (no. 2015-12-03/06).*

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