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Glycyrrhizic acid suppresses inflammation and reduces the increased glucose levels induced by the combination of *Porphyromonas gulae* and ligature placement in diabetic model mice

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ABSTRACT

Diabetic patients are at an increased risk of developing severe and progressive periodontitis. Periodontal disease also increases the severity of diabetes by enhancing insulin resistance. Therefore, the regulation of periodontal inflammation in diabetic patients may contribute to the control of both diseases. Glycyrrhizic acid exerts anti-inflammatory effects by inhibiting high mobility group box 1 (HMGB1). HMGB1, one of the ligands of the receptor for advanced glycation end products (RAGE), is a damage-associated molecular pattern and induces inflammatory cytokine production. In the present study, we examined the effects of glycyrrhizic acid on ligature- and *Porphyromonas gulae* infection-induced periodontitis as well as the involvement of the HMGB1-RAGE axis in diabetic model mice.

The molars of diabetic model mice, established by feeding HFD32 to KK/TaJcl mice, were subjected to silk thread ligation and *P. gulae* was then intraorally applied in the presence or absence of glycyrrhizic acid given topically. The topical application of glycyrrhizic acid suppressed ligature/*P. gulae*-induced increases in interleukin (IL)-6 and tumor necrosis factor (TNF)-α at the mRNA level in the gingiva and at the protein level in serum. Furthermore, glycyrrhizic acid suppressed ligature/*P. gulae*-induced increases in serum amyloid A (SAA) in serum and fasting blood glucose levels. It also suppressed ligature/*P. gulae*-induced increases of HMGB1 and RAGE at the mRNA level in the gingiva and at the protein level in serum. A mouse anti-HMGB1-neutralizing antibody inhibited increases in serum glucose levels. In conclusion, topical treatments with glycyrrhizic acid may suppress periodontal and systemic inflammation and reduce blood glucose levels through the HMGB1-RAGE axis in diabetic mice.

1. Introduction

Periodontal disease is a bacterial biofilm-associated inflammatory disease caused by the colonization of the gingival sulcus by periodontopathogenic bacteria. Periodontal disease is associated with several systemic inflammatory diseases, including diabetes, cardiovascular diseases, rheumatoid arteritis and dementia. A reciprocal relationship is known to exist between periodontal disease and diabetes. Periodontal disease has been described as the sixth complication of diabetes [1] and diabetic patients are at an increased risk of developing severe and progressive periodontitis [2–4]. Impairments in a host's defense system to invading bacterial pathogens and excessive inflammatory responses in patients with diabetes may contribute to more severe periodontal diseases [5]. Periodontal disease also increases the severity of diabetes by enhancing insulin resistance. Various inflammatory cytokines produced by inflamed periodontal tissue, activated macrophages or monocytes, may enter the systemic circulation and be transported to distant tissues. Adipocytes stimulated by these factors actively secrete inflammatory cytokines, such as IL-6, resulting in excessive inflammatory responses, which enhance insulin resistance and increase

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Table 1
Effects of feeding HFD32 on KK/TaJcl mice.

	Weight (g)	Weight gain (g)	Food intake/week (g)	Calorie intake/week (kcal)
Control (n = 12)	32.96 ± 2.26	7.07 ± 0.70	30.49 ± 1.22	109.79 ± 4.41
HFD32 (n = 12)	44.25 ± 3.74**	17.73 ± 1.26**	31.22 ± 4.64	158.46 ± 22.45**
P value	< 0.01	< 0.01	0.95	< 0.01

Differs significantly from the control (Mann-Whitney U test, **P < 0.01).

glucose levels [6–8].

Sporadically elevated levels of blood glucose in diabetes may induce the generation of largely irreversible advanced glycation end-products (AGEs). AGEs accumulate in the tissues and plasma of humans and rodents with normal aging; however, their rate of accumulation is increased in diabetes as a result of prolonged hyperglycemia. Receptor for AGE (RAGE) is a central cell-surface receptor for AGE and a multi-ligand member of the immunoglobulin superfamily of cell-surface molecules. RAGE is expressed on many cell types, including endothelial cells, monocytes/macrophages, lymphocytes, dendritic cells, and fibroblasts, and its expression is weak under physiological conditions [9], but increases when ligands, such as AGEs, accumulate [10,11]. Therefore, RAGE is strongly expressed and plays an important role in the pathogenesis of diabetic complications [12,13].

High mobility group box 1 (HMGB1) is an intranuclear highly conserved non-histone chromosomal protein that functions as a stabilizer of the nucleosome structure and regulator of gene transcription [14,15]. It may be released into the extracellular milieu from immune and non-immune cells in response to various stimuli. Extracellular HMGB1 contributes to the pathogenesis of numerous chronic inflammatory and autoimmune diseases, including sepsis [16], rheumatoid arthritis [17], atherosclerosis [18], chronic kidney disease [19], and periodontal disease [20,21]. HMGB1 binds to RAGE and induces a series of inflammatory responses [22]. Since RAGE is strongly expressed in the gingiva of diabetic patients with periodontitis [23,24], the HMGB1-RAGE axis may play a crucial role in the reciprocal relationship between periodontal disease and diabetes [25]. Therefore, the regulation of HMGB1-associated inflammation in gingival tissue may result in the suppression of the negative spiral that leads to the progression of periodontal disease in diabetes.

Glycyrrhizic acid, the main active compound in licorice, is known to exhibit pharmacological activities, including anti-inflammatory or antioxidative effects. Glycyrrhizic acid has been reported to inhibit the release of HMGB1 from activated or injured cells [26,27]. It has been suggested to bind to HMGB1 and inhibit its cytokine-like activities [28]. An anti-HMGB1-neutralizing antibody was recently shown to attenuate periodontal inflammation and bone resorption in a murine periodontitis model [29]. Based on these findings, we propose that glycyrrhizic acid may improve periodontal disease and serum glucose levels by suppressing gingival inflammation in diabetic subjects.

Porphyromonas gulae is a periodontal pathogen of canines and felines and is associated with the onset and progression of chronic periodontitis [30–32]. *P. gulae* has similar virulence and immunological characteristics to those of the human periodontal pathogen *Porphyromonas gingivalis* [33,34]. Since *P. gulae* infection seems to be capable of colonizing and eliciting alveolar bone loss more than *P. gingivalis* infection following oral challenge in mice [31], it may be considered that *P. gulae* infection induces a prominent inflammation in murine periodontitis model. Accordingly, we used *P. gulae* as periodontal pathogen instead of *P. gingivalis*. In the present study, we examined the effects of topical application of glycyrrhizic acid on inflammation and enhanced serum glucose levels induced by the combination of ligature placement and *P. gulae* infection in high fat diet-induced diabetic mice.

2. Materials and methods

2.1. Animals

KK/TaJcl mice (Nihon Clea Tokyo, Japan) were housed under climate-controlled conditions with a 12:12-h light-dark cycle. All protocols were approved by the Institutional Review Board of Hiroshima University (A16-26-2).

2.2. Establishment of diabetic model mice

Four-five-week-old male KK/TaJcl mice were fed a normal or high-fat diet (HFD32, Nihon Clea) for 7 weeks. Body weights were monitored and food intake was recorded by subtracting the amounts of food left in the cage from the weekly amounts given. The high-fat diet group developed obesity with an increased calorie intake (Table 1). The fasting blood glucose level test and oral glucose tolerance test (OGTT) revealed an abnormality in glucose tolerance in mice fed the high-fat diet (Table 2).

2.3. Culture of P. gulae

P. gulae (ATCC 51700) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured on a sheep blood agar plate using the Anaeropack system. After a 2-day incubation, *P. gulae* was inoculated into 40 ml of 3% trypticase soy broth, 0.5% yeast extract, hemin (2.5 mg), and vitamin K3 (25 mg). Bacteria were harvested in the exponential growth phase and washed with phosphate-buffered saline (PBS).

2.4. Ligature placement and P. gulae infection in diabetic model mice

After the diabetic mice were established, the ligature placement and *P. gulae* application were treated with the diabetic mice. The left maxillary second molar of diabetic mice was ligated with 5–0 silk thread under anesthesia. *P. gulae* was orally applied at 1×10^8 CFU in 2% carboxymethyl cellulose gel twice a week throughout the experimental period.

2.5. Application of glycyrrhizic acid

Glycyrrhizic acid ($C_{42}H_{60}K_2O_{16}$) was supplied from Maruzen Pharmaceuticals Co, LTD (Hiroshima, Japan). The purity of glycyrrhizic acid from licorice is 96.0–102.0%. After the diabetic mice were

Table 2
OGTT after 7 week of feeding HFD32 on KK/TaJcl mice. 1 g/kg of D-Glucose was orally injected for OGTT.

Minute	0	15	30	60	90
Control (n = 5) (mg/dL)	154.8 ± 27.11	313.8 ± 34.16	305.8 ± 27.65	244 ± 44.03	228.8 ± 36.91
HFD32 (n = 5) (mg/dL)	342 ± 37.26**	481.8 ± 81.37**	489 ± 65.93**	460 ± 64.54**	429.6 ± 64.51**
P value	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

established, to examine the preventive effect of glycyrrhizic acid, a total of 1%/w of glycyrrhizic acid dissolved in 3% hydroxyethyl cellulose gel was orally applied at 1 ml/kg every day for 2 or 4 weeks in the presence or absence of ligature treatment and *P. gulae* infection.

2.6. Administration of an anti-HMGB1 antibody and control IgG

An anti-HMGB1 antibody (50 μg/mice, Sigma Aldrich, St. Louis, MO, USA) and control IgG (Santa Cruz, Dallas, TX, USA) were administered *via* the tail vein at the beginning of fasting.

2.7. Histological analysis

Mice were sacrificed 2 weeks after ligature placement. Tissue samples were resected en bloc from the left molar region and fixed with 10% paraformaldehyde solution. They were then decalcified in 10% ethylenediaminetetraacetic acid (EDTA) solution in PBS at 4 °C for 14 days. Decalcified tissue blocks were embedded in paraffin. Sections (thickness of 5 μm) of the frontal plane parallel to the long axis of the tooth, including the root apex, were cut and collected on glass slides. Sections were stained with hematoxylin and eosin, and observed under a light microscope.

2.8. Serum antibody for P. gulae

Serum IgG antibody titers against *P. gulae* were measured by using ELISA. *P. gulae* bacterial solution (10^8 CFU/ml) was used as an antigen. Blocking was performed with 1% bovine serum albumin (Sigma Aldrich). One hundred-fold diluted serum was used as the primary antibody, and a peroxidase-labeled goat anti-mouse IgG antibody (R&D systems, Minneapolis, MN, USA) diluted 1,000 fold was used as the secondary antibody. H_2O_2 (Sigma Aldrich) and *o*-phenylenediamine (Thermo Fisher Scientific, Waltham, MA, USA) were mixed in Na_2HPO_4 (Wako, Osaka, JAPAN) solution as substrate solution. $2NH_2SO_4$ was added to stop the reaction. Absorbance at 450 nm was measured using an iMark microplate reader (BIO RAD, Hercules, CA, USA). Serum collected at the start of the experiment in each group was compared with that collected 2 weeks after the start of the experiment.

2.9. Fasting glucose

Eight-hour fasting blood glucose concentrations were assessed by the Glucose Pilot assay (Aventir Biotech, Carlsbad, CA, USA) 4 weeks after the start of the experiment.

2.10. Detection of C-reactive protein (CRP), interleukin (IL)-6, tumor necrosis factor (TNF)-α, serum amyloid A (SAA), HMGB1, and RAGE

The eBioscience Mouse IL-6 platinum ELISA kit (Thermo Fischer Scientific), Quantikine Mouse TNF-α ELISA kit (R&D), Quantikine Mouse CRP ELISA kit (R&D), PHASE Murine Serum Amyloid A Assay (Tridelta Development, Maynooth, Irelamd), HMGB1 detection kit (Chondrex, Redmond, WA, USA), and Mouse RAGE ELISA kit (Abcam, Cambridge, UK) were used to measure the concentrations of CRP, IL-6, TNF-α, SAA, HMGB1, and RAGE in serum 2 weeks after the start of the experiment.

2.11. Culture of murine gingival fibroblasts

Murine gingival fibroblasts were collected from 4-week-old male KK/Tajcl mice. Fibroblasts were pre-cultured in DMEM-low glucose medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/ml penicillin (Sigma Aldrich), and 100 mg/ml streptomycin (Sigma Aldrich) in 24-well plates at 37 with 5% CO_2. At semi-confluence, cells were washed and cultured in antibiotic-free DMEM-low glucose medium with 10% FBS. Confluent murine gingival fibroblasts were pretreated with 100 μM glycyrrhizic acid for 1 h, then, exposed to 2×10^6 CFU of *P. gulae* for 24 h before end of incubation.

2.12. Small interfering RNA (siRNA) knockdown of HMGB1

Validated HMGB1 siRNA and negative-control siRNA were obtained from Invitrogen (Van Allen Way Carlsbad, CA). Murine gingival fibroblasts were cultured in DMEM-low glucose medium supplemented with 10% FBS 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C for 24 h. One hundred nanomoles of HMGB1 siRNA and negative control siRNA were transfected into cells using Lipofectamine 3000 transfection regent (Invitrogen), according to the manufacturer's instructions. Cells treated with or without stimulants were collected after a 24-h incubation.

2.13. Real-time PCR

In animal study, gingival tissue from the mandible, adipose tissue, and liver tissue were dissected 2-weeks after the start of the ligation. *In vitro* study, confluent cells were collected at the end of incubation. Total RNA was extracted using RNA iso plus (Takara Bio, Shiga, JAPAN) and quantified by spectrometry at 260 and 280 nm. First-standard cDNA synthesis was performed with 1 μg of total RNA extract in a total volume of 20 μl using ReverTra Ace (TOYOBO, Osaka, JAPAN). Real-time PCR was performed with STEP ONE PLUS-D (Applied Biosystems, Foster City, CA, USA). Sense and anti-sense primers for mouse IL-6, IL-1β, TNF-α, RAGE, HMGB1, and GAPDH mRNA are listed in Table 3.

2.14. Minimum inhibitory concentration (MIC) for P. gulae

The MICs of glycyrrhizic acid were determined by microdilution methods. Briefly, after 24 h of incubation of *P. gulae* with glycyrrhizic acid at 37 °C, MICs were calculated by determining the minimum concentration at which no growth was visible.

2.15. Statistical analysis

The Mann-Whitney *U* test was used in two-group comparisons. The non-parametric Kruskal-Wallis test with the Mann-Whitney U test was used to compare more than two groups.

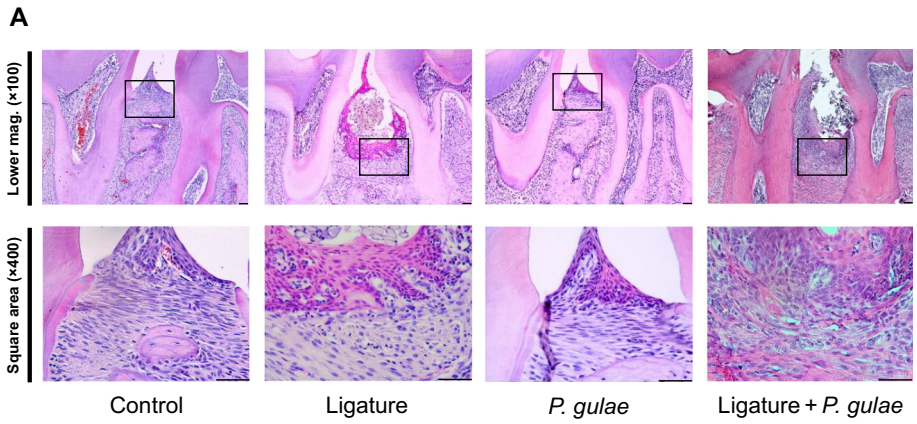
3. Results

3.1. Combination of ligature placement and P. gulae infection induced inflammation in gingival tissue of diabetic mice

Fig. 1-A indicates the preparation of murine gingival tissue divided into 4 groups: non-treated group (control), ligated group (ligature), *P. gulae* applied group (*P. gulae*), and the combination of ligature

Table 3
Primers for real-time PCR used in the present study.

Gene		Sequence (5′-3′)
IL-6	Forward	GAAATGAGAAAAGAGTTGTGCAATGG
	Reverse	ATATCCAGTTTGGTAGCATCCATCAT
IL-1β	Forward	CCTTGTGCAAGTGTCTGAAGC
	Reverse	TCATCTTTTGGGGTCCGTCAAC
TNF-α	Forward	GGTGCCTATGTCTCAGCCTCTT
	Reverse	ATTTGGGAACTTCTCATCCCTTTGG
HMGB1	Forward	GCAAAGAAACTAGGAGAGATGTGGAA
	Reverse	CTCTGTAGGCAGCAATATCCTTCTC
RAGE	Forward	GATCCTGCCTCTGAACTCACA
	Reverse	CTTCCTTCACGAGTGTTTCTTTG
GAPDH	Forward	CCTGGAGAAACCTGCCAAGTATG
	Reverse	TGTTGCTGTAGCCGTATTCATTGT



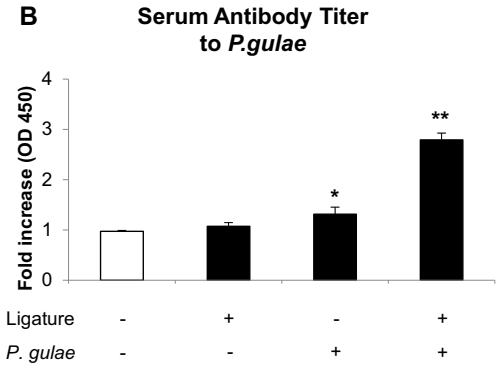


Fig. 1. Induction of periodontitis in diabetic mice by the ligature/*P. gulae* treatment. (A) Histological findings in periodontal tissues. Hematoxylin & eosin staining. 100× magnification and 400× magnification of periodontal tissue representing the control group, ligature group, *P. gulae* group and ligature/*P. gulae* group. (B) Immunoglobulin (Ig)G titer against *P. gulae* in the serum of the control group, ligature group, *P. gulae* group and ligature/*P. gulae* group ($n = 4$). Values are means ± SD. Differs significantly from the control (Non-parametric Kruskal-Wallis test with the Mann-Whitney *U* test, $^{*}P < 0.05$, $^{**}P < 0.01$).

placement and *P. gulae* group (ligature/*P. gulae*). A histological analysis showed that ligature/*P. gulae* resulted in the destruction of periodontal tissue, including the gingival epithelium and connective tissue (Fig. 1A). Moreover, the highest serum antibody titer to *P. gulae* was observed in ligature/*P. gulae*-treated mice (Fig. 1B). Therefore, we decided to use ligature/*P. gulae* group as periodontitis model in the following experiment. Ligature/*P. gulae* increased the mRNA levels of IL-6, IL-1β, and TNF-α in the gingiva of diabetic mice, but had a negligible effect on these levels in liver and adipose tissues (Fig. 2A, B, C). Furthermore, ligature/*P. gulae* up-regulated IL-6, TNF-α, CRP, and SAA levels in serum and enhanced fasting blood glucose levels in diabetic mice (Fig. 2D, E).

3.2. Glycyrrhizic acid suppressed ligature/*P. gulae*-induced increases in inflammatory cytokines in gingival tissue and serum in diabetic mice

Topical application of glycyrrhizic acid suppressed ligature/*P. gulae*-induced increases in IL-6, IL-1β, and TNF-α in the gingiva of diabetic mice (Fig. 3A). Furthermore, glycyrrhizic acid down-regulated ligature/*P. gulae*-induced increases in IL-6, TNF-α and SAA levels in serum and fasting glucose (Fig. 3B, C). However, in the absence of periodontal model, glycyrrhizin acid had little effect on these levels (Fig. 3B, C). It also down-regulated ligature/*P. gulae*-induced increases in the serum antibody titer for *P. gulae* (Fig. 3D).

3.3. Involvement of RAGE and HMGB1 in regulation by glycyrrhizic acid

Glycyrrhizic acid suppressed ligature/*P. gulae*-induced increases in HMGB1 and RAGE at the mRNA level in the gingiva of diabetic mice (Fig. 4A). It also down-regulated ligature/*P. gulae*-induced increases in HMGB1 and RAGE in serum (Fig. 4B). The mouse anti-HMGB1-neutolizing antibody inhibited increases in serum glucose levels, whereas control IgG did not (Fig. 4C).

3.4. Glycyrrhizic acid suppressed *P. gulae*-induced increases of cytokines in murine gingival fibroblasts

The addition of *P. gulae* to the culture increased the expression of IL-6 and TNF-α at the mRNA level in murine gingival fibroblasts. In addition, the application of glycyrrhizic acid suppressed increases in the levels of these cytokines in gingival fibroblasts (Fig. 5A). Furthermore, the transfection of HMGB1 siRNA down-regulated the *P. gulae*-induced increase of IL-6 mRNA in murine gingival fibroblasts (Fig. 5B).

To evaluate the anti-microbial effects mediated by glycyrrhizin acid, MIC for *P. gulae* were examined. Glycyrrhizic acid at 0–100 μM did not show inhibitory effect (data not shown).

4. Discussion

In the present study, we demonstrated that the topical application of glycyrrhizic acid attenuated ligature/*P. gulae*–induced inflammation and the increases in glucose levels in serum in diabetic mice.

In the mice fed normal diet, the combination of ligature placement and *P. gulae* infection up-regulated the levels of CRP, IL-6, and fasting glucose and had little effect on SAA level in serum (Fig. S1). On the other hand, in the mice fed high-fat diet (diabetic mice), the combination of ligature placement and *P. gulae* infection increased the levels of SAA, IL-6, TNF-α and fasting blood glucose in serum and the increased levels are much higher than normal diet groups. Therefore, diabetic mice may be compromised host and subject to be influenced by inflammation.

In diabetic mice, the combination of ligature placement and *P. gulae* infection increased inflammatory cytokines and HMGB1 at the mRNA level in gingival tissue and at the protein level in serum, suggesting that periodontitis induced by ligature/*P. gulae* is HMGB1-associated inflammation. These results are consistent with previous findings obtained in animal studies that HMGB1 is expressed in response to

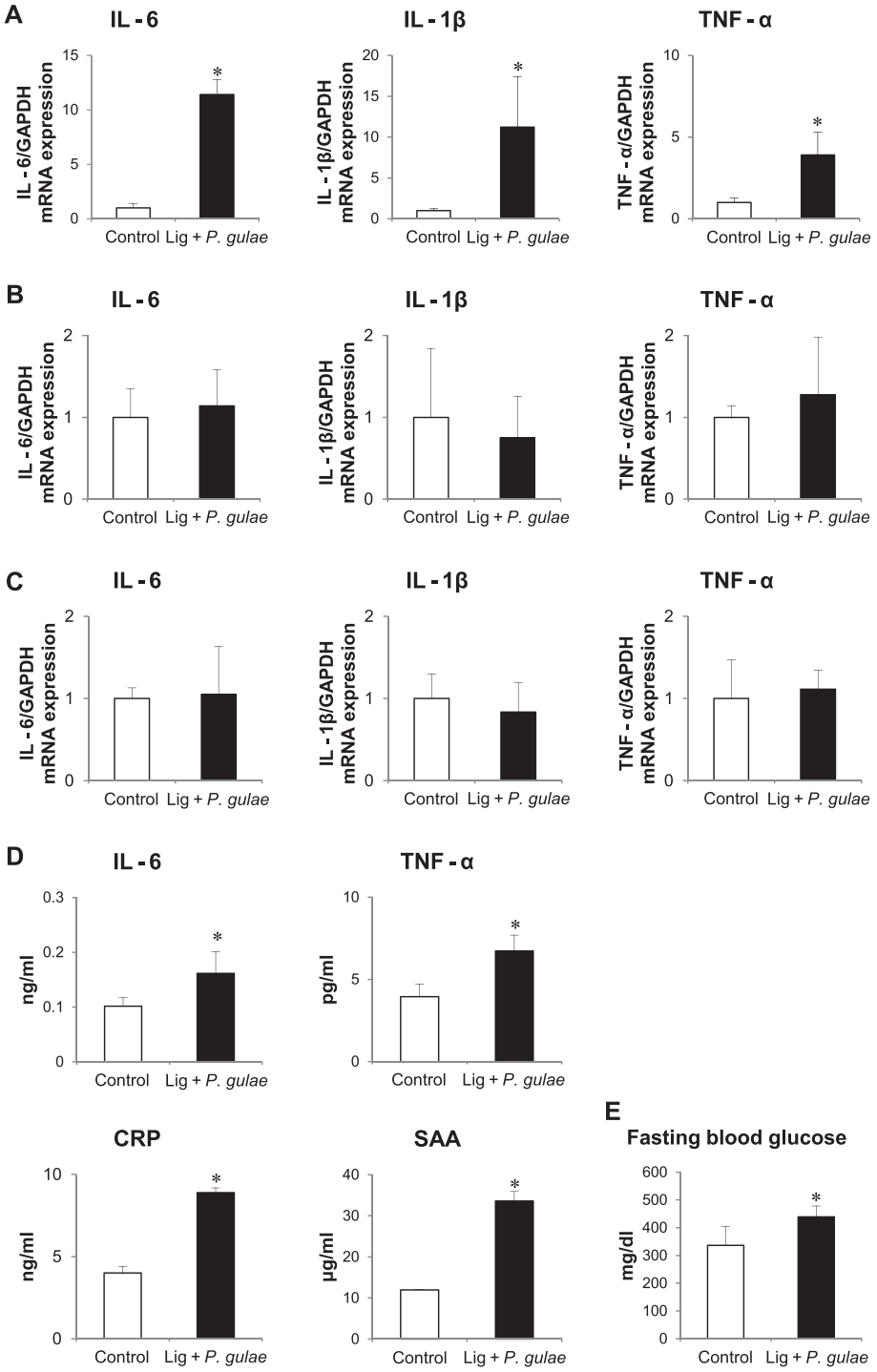


Fig. 2. Effect of the ligature/*P. gulae* treatment on inflammation in gingival, liver, and adipose tissues in diabetic mice. (A) Real-time PCR analysis of the mRNA of IL-6, IL-1β and TNF-α in gingival, (B) liver, and (C) adipose tissues ($n = 4$). (D) ELISA analysis of IL-6 ($n = 5$), TNF-α ($n = 5$), CRP ($n = 4$), and SAA ($n = 4$) levels in serum. (E) Fasting blood glucose levels ($n = 4$). Values are means ± SD. Differs significantly (Mann-Whitney U test, $^*P < 0.05$).

inflammatory stimuli caused by periodontal infection, which is crucial for the initiation of periodontitis [20,29]. Higher HMGB1 concentrations and more positive cells have been found in gingival crevicular fluid and inflamed gingival epithelial cell, respectively, in patients with periodontal disease than in healthy sites [35]. In addition, ligature/*P. gulae* increased RAGE at the mRNA level in gingival tissue and at the protein level in serum in diabetic mice. This is supported by previous findings showing that the amount of RAGEs was influenced by the accumulation of inflammatory mediators, such as HMGB1 [13]. In addition to the AGE-RAGE complex, the HMGB1-RAGE complex may play a role in the progression of periodontal disease in diabetic patients. For example, increased levels of RAGE and HMGB1 have been reported in the retinas of diabetic patients and rat models with retinopathy [36,37]. Therefore, the blockade of the HMGB1-RAGE axis is a promising therapeutic strategy for the management of periodontal disease in diabetic patients. It has already been demonstrated that intravenously injected glycyrrhizic acid suppresses traumatic brain injury by reducing the HMGB1-RAGE interaction in rats [38].

In the present study, we showed, for the first time that the topical application of glycyrrhizic acid suppressed the increased expressions of

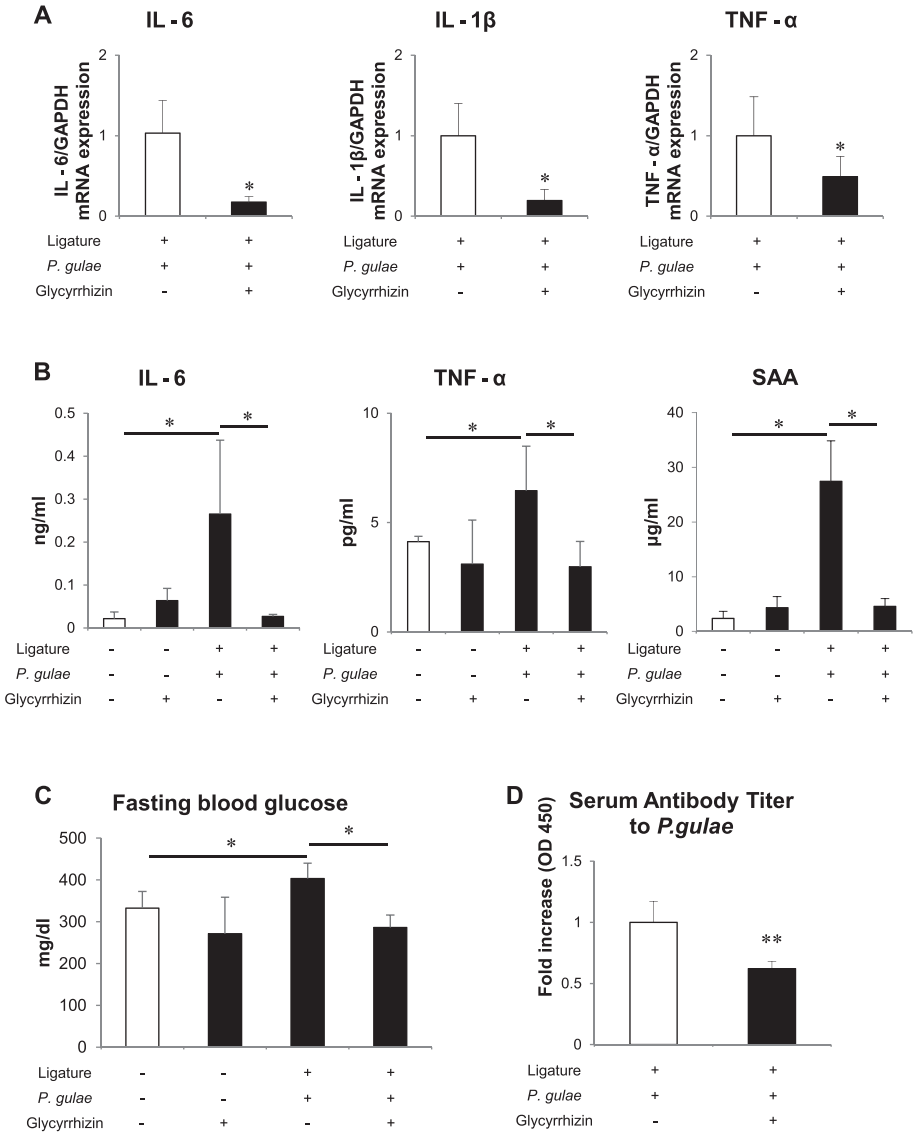


Fig. 3. Effects of topical treatment of glycyrrhizic acid on cytokine levels in the gingiva and serum and fasting blood glucose levels in ligature/*P. gulae*-treated diabetic mice. (A) Real-time PCR analysis of the mRNA of IL-6 ($n = 4$), IL-1β ($n = 4$) and TNF-α ($n = 5$) in the gingiva. (B) ELISA analysis of IL-6 ($n = 4$), TNF-α ($n = 5$), and SAA ($n = 5$) levels in serum. (C) Fasting blood glucose level ($n = 5$). (D) The IgG titer against *P. gulae* in serum ($n = 4$). Values are means ± SD. Differs significantly (Mann-Whitney U test, $^{*}P < 0.05$, $^{**}P < 0.01$).

inflammatory cytokine, HMGB1, and RAGE in gingival tissue with ligature/*P. gulae*-induced gingival inflammation. Furthermore, glycyrrhizic acid suppressed the increases in inflammatory cytokines and SAA as well as HMGB1 and RAGE at the protein level in serum. These results imply that glycyrrhizic acid suppressed HMGB1-associated gingival inflammation and the subsequent amplification of inflammatory cytokines in serum. Previous studies demonstrated that intravenously injected glycyrrhizic acid inhibited extracellular HMGB1 cytokine activity in experimental animals and protected the spinal cord, liver, brain, and myocardium against ischemia-reperfusion (I/R)-induced injury [39]. Glycyrrhizic acid has also been used clinically to treat patients with chronic hepatitis in China and Japan and exerts a satisfactory therapeutic effects on many other diseases. A number of clinical studies reported that intravenously injected glycyrrhizic acid was an effective treatment for various types of inflammation (mainly in the liver, but also in the lungs, kidneys, intestines, and spinal cord) [40]. Collectively, the present results suggest that the topical application of glycyrrhizic acid is clinically applicable to the regulation of gingival inflammation as a preventive medicine for periodontal disease in diabetes.

In the present study, glycyrrhizic acid and the anti-HMGB1-neutlizing antibody improved glucose levels, suggesting that glycyrrhizic acid influences blood glucose levels by suppressing inflammation and insulin resistance. Similar findings showed that the inhibition of HMGB1 protected the retina against ischemia-reperfusion and reduced insulin resistance protein levels [41]. Another study demonstrated that metformin, an anti-diabetic drug with antioxidant properties, protected against hyperglycemia-induced cardiomyocyte injury by inhibiting the expression of RAGE and HMGB1 [42]. Thus, the control of inflammation by the down-regulation of HMGB1 may improve insulin resistance and blood glucose levels. A previous study reported that glycyrrhizic acid itself exerted anti-diabetic effects [43]. The mice fed glycyrrhizin diet had lower blood glucose insulin levels in diabetic KK-Ay mouse [44]. Therefore, glycyrrhizic acid appears to suppress the negative spiral leading to the progression of periodontal disease and diabetes.

Increased cytokine levels in the serum of diabetic subjects with periodontal disease are thought to be caused by an interaction between enlarged adipocytes and activated macrophages from gingival tissue. Dental infection with *P. gingivalis* increased the mRNA levels of

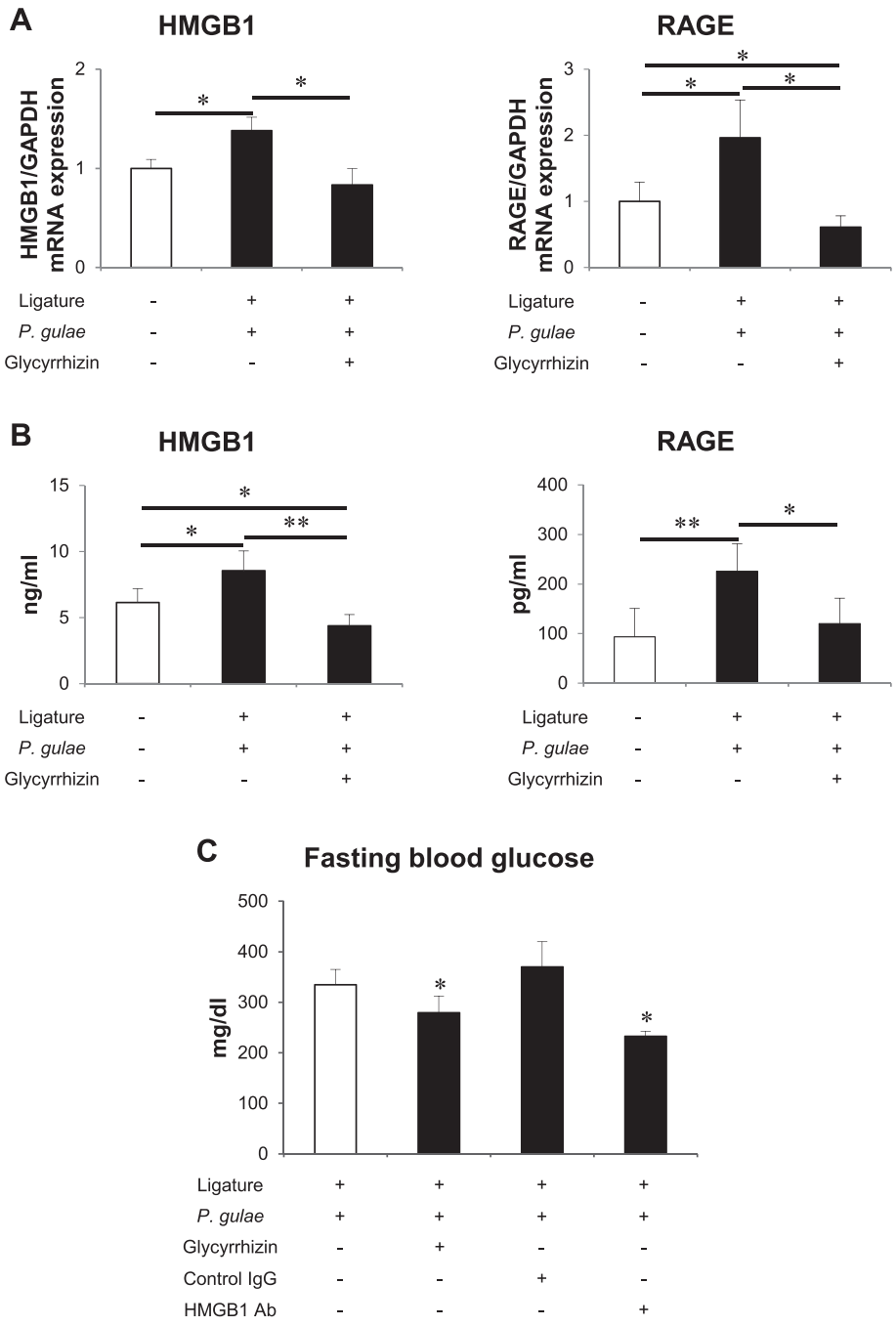


Fig. 4. Involvement of the HMGB1/RAGE complex on the glycyrrhizic acid-induced suppression of fasting glucose levels. (A) Effect of glycyrrhizic acid on the mRNA expressions of HMGB1 and RAGE in the gingiva. Real-time PCR analysis of the mRNAs of HMGB1 and RAGE in the gingiva ($n = 4$). (B) Effect of glycyrrhizic acid on the levels of HMGB1 and RAGE in serum. ELISA analysis of HMGB1 and RAGE in serum ($n = 6$). (C) Effects of the anti-HMGB1 antibody on fasting blood glucose levels ($n = 4$). Values are means ± SD. Differs significantly from the control (Non-parametric Kruskal-Wallis test with the Mann-Whitney U test, $^{*}P < 0.05$, $^{**}P < 0.01$).

inflammatory cytokines in steatotic hepatocytes [45]. However, in the present study, the combination of ligature placement and *P. gulae* infection had a negligible effect on inflammatory cytokines at the mRNA level in liver and adipose tissues despite increases in CRP, SAA, and IL-6 levels in serum. These results imply that the increases observed in cytokine levels in the serum of this model are amplified in other tissues, for example, the endothelial cells of vessels, but not adipose tissue or liver tissue.

In the *in vitro* study, the addition of glycyrrhizic acid to the culture of murine gingival fibroblasts suppressed increases in the levels of inflammatory cytokines by *P. gulae*. On the other hand, glycyrrhizic acid did not inhibit the *P. gulae*-induced increases in inflammatory cytokines in a culture of RAW 264.7 cells (unpublished data). In addition, the transfection of HMGB1 siRNA partially down-regulated HMGB1 mRNA in murine gingival fibroblasts. The partial knockdown of HMGB1 inhibited the *P. gulae*-induced increase of IL-6 mRNA, suggesting that HMGB1 play an important role in the *P. gulae*-induced increase of IL-6. Glycyrrhizic acid is known to inhibit HMGB1 activation [28]. Therefore, it is considered that glycyrrhizic acid is able to inhibit *P. gulae*-induced increase of IL-6 by down-regulating HMGB1 activation. Although the regulatory mechanism of cytokine expressions used by glycyrrhizic acid needs to be elucidated in more detail, the present results suggest that HMGB1 from gingival fibroblasts is one of the targets of glycyrrhizic acid for the down-regulation of *P. gulae*-induced inflammation.

The present results suggest that glycyrrhizic acid down-regulated inflammatory responses in gingival tissue and improved glucose levels by suppressing circulating cytokine levels.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.045.

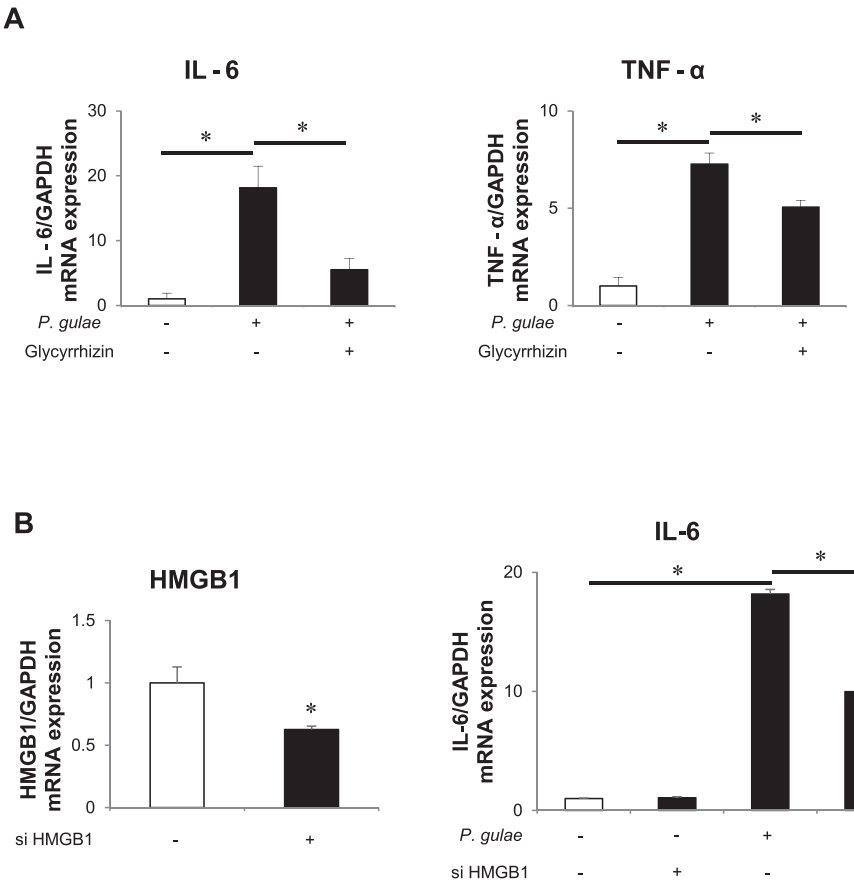


Fig. 5. Effects of glycyrrhizic acid on IL-6 and TNF-α at mRNA levels in a culture of murine gingival fibroblasts. (A) The mRNA expressions of IL-6 and TNF-α in murine gingival fibroblasts stimulated by *P. gulae*, were examined by real-time PCR ($n = 3$). (B) Murine gingival fibroblasts, having transfected with negative control and HMGB1 siRNA, were stimulated by *P. gulae*, and then the mRNA expressions of HMGB1 and IL-6 were examined by real-time PCR ($n = 4$). Values are means ± SD. Differs significantly (Non-parametric Kruskal-Wallis test with the Mann-Whitney U test, $^*P < 0.05$).

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Conflict of interest

SK is employee of Kobayashi Pharmaceutical Co., Ltd.

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