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Assessing the therapeutic potential of *Ganoderma lucidum* spore oil in alleviating periodontal tissue damage in murine periodontitis model

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Abstract

Periodontal disease presents a significant challenge in oral health due to its chronic inflammatory nature and subsequent degradation of tooth-supporting structures. Natural compounds have attracted attention for their potential therapeutic effects in alleviating symptoms of periodontitis (PD). In this study, we investigated the impact of *Ganoderma lucidum* spore oil (GLSO), a lipid component extracted from broken-walled GLS using the supercritical CO_2 extraction method, on PD pathogenesis in vitro and in vivo. Treatment of human gingival fibroblasts with GLSO resulted in a significant reduction in the expression of inflammatory factors, including matrix metalloproteinase (MMP)-1 and interleukin (IL)-8, upregulated by lipopolysaccharide or IL-1β. Molecular mechanism studies revealed that the observed decrease in inflammatory factor expression may be attributed to the inhibition of phosphorylated c-Jun N-terminal kinase activity by GLSO. Furthermore, intraperitoneal injection of GLSO in a ligature-induced PD mouse model led to a notable reduction in periodontal inflammation and alveolar bone loss, accompanied by decreased levels of MMP-1 and IL-8. These in vivo results support the potential therapeutic efficacy of GLSO in alleviating PD symptoms. Overall, our study provides novel insights into the beneficial effects of GLSO in PD management. Further research is warranted to elucidate the underlying molecular mechanisms and explore the clinical applicability of GLSO as a promising therapeutic agent for PD treatment.

Keywords Periodontitis, Gingival fibroblasts, Alveolar bone loss, Ganoderma lucidum spore oil, Inflammation

Introduction

Periodontal disease, a prevalent chronic inflammatory condition affecting the oral cavity, arises from a complex interplay of microbial dysbiosis and host immune response dysregulation [1]. Microbial pathogens colonize dental plaque, triggering an inflammatory cascade characterized by the release of pro-inflammatory mediators and the recruitment of immune cells, leading to the destruction of periodontal tissues [2]. Gingivitis, the initial stage of periodontal disease, manifests as gingival inflammation and bleeding upon probing, primarily caused by the accumulation of dental plaque along the

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gingival margin. If left untreated, gingivitis can progress to periodontitis (PD), wherein the inflammatory process extends beyond the gingiva to involve deeper periodontal structures, including the periodontal ligament and alveolar bone, resulting in irreversible tissue damage and tooth loss [3]. The global burden of periodontal disease is substantial, with a significant impact on oral health-related quality of life and systemic health outcomes. Epidemiological studies have highlighted the high prevalence of periodontal disease across diverse populations, with estimates suggesting that over 50% of adults worldwide suffer from some form of periodontal disease [4]. Moreover, periodontal disease is not merely a local oral health issue but has also been linked to various systemic conditions, including diabetes mellitus, cardiovascular diseases, respiratory infections, and adverse pregnancy outcomes, underscoring its systemic implications and the need for comprehensive management strategies [3].

Current treatment modalities for periodontal disease primarily aim to arrest disease progression, reduce inflammation, and promote periodontal tissue regeneration [5]. Mechanical debridement, including scaling and root planing, remains the cornerstone of periodontal therapy, aimed at removing bacterial biofilm and calculus from tooth surfaces and root surfaces [6]. Adjunctive pharmacotherapy, such as locally administered antimicrobials, systemic antibiotics, and anti-inflammatory agents, may be employed to supplement mechanical therapy and control inflammation [7, 8]. However, these conventional approaches have several limitations, including variable treatment outcomes, microbial resistance, adverse effects, and challenges in achieving long-term disease control and tissue regeneration [9]. Furthermore, the effectiveness of periodontal therapy is influenced by various patient-related factors, such as oral hygiene practices, smoking status, genetic predisposition, systemic health conditions, and socioeconomic factors [10]. Poor patient compliance, inadequate access to dental care, and disparities in healthcare access further compound the challenges in managing periodontal disease effectively [11]. Consequently, there is a critical need for alternative therapeutic approaches that address the multifactorial nature of periodontal disease and offer improved outcomes for patients.

In recent years, there has been growing interest in exploring the therapeutic potential of natural products derived from medicinal plants in the management of periodontal disease. Various plant-derived compounds, such as polyphenols, flavonoids, alkaloids, and essential oils, have been investigated for their anti-inflammatory, antimicrobial, and antioxidant properties, which may confer beneficial effects on periodontal health [12]. For example, curcumin, a bioactive compound found in turmeric, has been shown to exhibit potent anti-inflammatory and antioxidant activities, attenuating gingival inflammation and alveolar bone loss in experimental models of periodontitis [13]. Similarly, green tea catechins, particularly epigallocatechin-3-gallate (EGCG), have demonstrated anti-inflammatory and antimicrobial effects, inhibiting periodontal pathogens and suppressing inflammatory cytokine production in periodontal tissues [14]. Moreover, in our previous study, we investigated the anti-inflammatory effects of Avn-C in human gingival cells and the its protective role on alveolar bone erosion in PD experimental mice model [15].

Ganoderma lucidum spores (GLS) are minute germ cells discharged from the mushroom cap during mature stage of growth. GLS have a higher bioactivity than *Ganoderma lucidum* [16] and have been recognized as a promising drug due to their versatile biological activity [17]. The physiologically active substances GLS include polysaccharides, triterpenoids, peptides, amino acids, and fatty acids [18–20]. These biologically active substances of GLS have a wide range of beneficial effects, including anti-tumor, nervous system protection, immunity improvement, anti-ageing, hepatoprotection, and treatment of metabolic diseases [21]. *Ganoderma lucidum* spore oil (GLSO) is an extract primarily composed of lipids, extracted from broken-walled GLS using supercritical CO_2 extraction method. It has emerged as a potential adjunctive therapy for managing various disease [22]. Previous studies have demonstrated that GLSO possesses a myriad of pharmacological properties, including anti-inflammatory [23], anti-oxidant [24], immunomodulatory [25], and anti-microbial [26] effects, which may confer therapeutic benefits in the context of periodontal disease. However, despite the promising preclinical evidence supporting the efficacy of GLSO in alleviating inflammation and tissue damage in various inflammatory conditions, its specific effects on periodontal tissues and disease outcomes remain to be elucidated.

Given the limitations of current treatment modalities and the potential of GLSO as an adjunctive therapy, there is a compelling rationale for further investigation into the therapeutic potential of GLSO in periodontal disease management. Therefore, the present study aims to explore the effects of GLSO on periodontal inflammation and alveolar bone resorption using in vitro cell culture models and in vivo animal models of ligature-induced PD. By elucidating the underlying mechanisms of GLSO action and addressing the limitations of current treatment modalities, this research may provide valuable insights into the development of novel adjunctive therapies for the prevention and treatment of periodontitis, ultimately improving oral health outcomes and overall well-being.

Materials and methods

Extraction of GLSO using supercritical CO_2

The extraction of GLSO was conducted using the supercritical CO_2 extraction process as previously described [27, 28]. The extraction system consisted of a 5 L extractor, two separators (3 L for separator 1 and 2 L for separator 2), a chiller, and a high-pressure CO_2 pump. After loading 2 kg of GLS powder into the extractor, CO_2 was pumped into the system. Following purging with CO_2 three times to remove air, valves were closed except for V1 and V2 to allow CO_2 to enter the extractor. Circulation and dynamic extraction occurred once the pressure and temperature inside reached 40 MPa and 55 °C, respectively. Separator 1 and 2 maintained temperatures of 50 °C and pressures of 13 MPa, and 40 °C and 5 MPa, respectively. With a CO_2 mass flow rate of 35 kg/h, extraction lasted for 2 h. Finally, GLSO was collected via valve V7 for storage and analysis.

Human gingival fibroblasts primary cell culture and GLSO treatment

Human gingival tissues were obtained from extracted teeth of healthy individuals undergoing orthodontic treatment. Prior to tissue collection, written informed consent was obtained from all participants, and the study protocol received approval from the Institutional Review Board of Chonnam National University Hospital (CNUDH-2023-010). Gingiva and ligament tissues at the middle third of the roots were obtained from extracted teeth and human gingival fibroblasts (hGFs) were isolated as previously described [29]. Briefly, gingival tissues collected from the extracted teeth were immediately rinsed 10 times in phosphate-buffered saline (PBS) containing 2% penicillin (Sigma-Aldrich, P3032) and 2% streptomycin (Gibco, 11860). The gingival tissues were minced and plated onto 100-mm culture dishes, then cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, 12800-017) supplemented with 10% fetal bovine serum (FBS; Capricorn, FBS-22A), 1% penicillin, and 1% streptomycin. hGFs were cultured at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 and used between passage 5 to passage 8 for experiments. hGFs were pre-treated with GLSO in serum-free medium for 30 min and stimulated with 5 μg/ml of *Porphyromonas gingivalis* lipopolysaccharide (LPS) (Inviogen, tlrl-pglps) or 2 ng/ml of human IL-1β (GenScript, Z02922-10) for 24 h. To confirm cellular pathways, cells were either treated with 2 ng/ml IL-1β at various time points (0, 0.25, 0.5, 1, 6, 12, 24 h) or co-treated with SP600125 (c-jun N-terminal kinase [JNK] inhibitor; Santa Cruz, SC-200635) and 2 ng/ml IL-1β for 24 h.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from primary cultured hGFs was isolated using TRIzol reagent (MRC, TR118). Subsequently, cDNA synthesis was performed via reverse transcription (Promega, A3803), followed by PCR (Geneall, 501-025). Quantitative RT-PCR was executed using SYBR Premix Ex Taq (Takara Bio, RR420A) and analyzed with a Quantstudio1 Real-Time PCR system (Thermo Fisher Scientific). All qRT-PCR reactions were conducted in duplicate, and fold changes were calculated using relative quantification methods with GAPDH as an internal control. Details relating to the primer used in the experiments are shown in Suppl Table 1.

Western blotting

hGFs were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM NaF, 1% NP-40, 0.5% deoxycholate, 0.2% SDS), supplemented with a protease inhibitor cocktail (Roche, 04906845001) and a phosphatase inhibitor cocktail (Roche, 11697498001), on ice for 30 min. Following centrifugation at 13,000 rpm for 30 min at 4 °C, the supernatant containing the lysate was collected, and protein concentrations were determined. The proteins were then separated on SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 h and subsequently incubated with the indicated primary antibodies for overnight, including anti-MMP-1 (Novus, NBP2-22123), anti-IL-8 (Cell signaling, #94407S), anti-pERK (Cell Signaling, #9101), anti-ERK (BD Biosciences, 610408), anti-pp38 (Cell Signaling, #9216), anti-p38 (Santa Cruz, sc-535), anti-pJNK (Cell Signaling, #9255), anti-JNK (Cell Signaling, #9252), anti-IκBα (Santa Cruz, sc-371), and anti-β-ACTIN (Sigma-Aldrich, A3854) as a loading control. Membranes were washed with 0.1% Tween 20 in Tris-buffered saline, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit IgG; Sigma-Aldrich, A6154 or anti-mouse IgG; Sigma-Aldrich, A6782). The blots were then detected using an ECL solution (Cytiva, RPN2235) and visualized by iBright1500 (Invivogen).

Immunofluorescence microscopy

The expression of MMP-1 and IL-8 was determined using an immunofluorescence (IF) assay. hGFs were pre-treated with 800 μg/ml GLSO in serum-free medium for 30 min and stimulated with 2 ng/m IL-1β for 24 h. After treatment, hGFs were fixed with 3.5% paraformaldehyde for 10 min at RT and rinsed with PBS three times. The cells were permeabilized with 0.1% Triton X-100 (Affymetrix, T1001) for 10 min and then washed three times.

Following this, the cells were incubated with 1% BSA for 30 min. Primary antibodies against MMP-1 (Abnova, PAB12708) or IL-8 (BIOSS, bs-0780R) were applied for 1 h at RT, followed by incubation with secondary antibodies conjugated with Alexa 488 (Life technology, A11029) and Alexa 594 (Life technology, A11037) for 1 h at RT. Cell nuclei were counterstained with DAPI for 10 min. The slides were mounted with Fluoroshield histology mounting medium (Sigma-Aldrich, F6182). Images were viewed under a Zeiss Axio Scope A1 microscope connected to a fluorescence component and analyzed by Adobe Photoshop software.

MMP activity assay

The MMP enzyme activity was determined using an assay kit (Abcam, ab112146) following the manufacturer's instructions. hGFs were pre-treated with GLSO in serum-free medium for 30 min, followed by 2 ng/ml IL-1β treatment for 24 h. MMP activity in the cultured medium was evaluated by adding 25 µl of cultured medium to 25 µl of assay buffer, incubating for 15 min at RT, and then adding 50 µl of the MMP green substrate working solution. After 1 h, fluorescence was measured using a microplate reader (Thermo Scientific, Fluoroskan FL) at Excitation/Emission = 488/525 nm.

Mouse studies

C57BL/6J male (9 weeks old) mice were purchased from Damool Science (Daejeon, Republic of Korea). Mice were housed at a constant temperature and relative air humidity with a 12 h light/dark cycle in pathogen-free barrier facilities at the Chonnam National University. For each experiment, age-matched mice were used and randomly allocated to each experimental group. They were fed with a normal chow diet and water ad libitum. We did not observe significant changes in body weights or gross morphological differences between mice in our study. This research was approved by the Animal Care and Ethics Committee of Chonnam National University.

Ligature-induced experimental PD models in mice

Ligature-PD model using male mice (10 weeks old C57BL/6J mice) was established via silk 5-0 (Ailee, SK521). The silk was tied around the right second molar in the maxilla and the left side was used as the control. GLSO (6 mg/kg body weight) was adjusted with polyethylene glycol-400 (PEG-400; Sigma-Aldrich, 8.07485) to a volume of 50 µl, according to the weight of the mouse [27]. 50 µl of GLSO or PEG-400 as a control was injected intraperitoneally (I.P.) every other day for 9 days after ligature. At 9 days after ligature, mice were sacrificed, and the maxillae were harvested for µ-CT scanning and histological analyses [30].

Micro-computed tomography (µ-CT) scan

All mice maxillae were collected as described in a previous study [31]. The collected tissues were fixed in 10% neutral buffered formalin (NBF; BBC biochemical, 0151) for overnight. The structure of alveolar bone was scanned by using the SkyScan 1172 µ-CT scanner with the following parameters 49 kV, 200 µA, 0.5 mm aluminum filter at 10.88 µm resolution. µ-CT data were analyzed the regions between the first and the second molar teeth in the buccal and lingual sides after drawing the region of interest (ROI), using CTAn (SkyScan) and Mimics 14.0 (Materialise).

Histology and immunohistochemistry

Fixed mice maxillae with 10% NBF were decalcified in 0.5 M EDTA (pH7.4; LPS solution, CBE002C) for 2 weeks. After dehydration, the tissues embedded in paraffin, and sectioned at 5 µm thickness. The paraffin sections were dehydrated and stained with hematoxylin and eosin (H&E; Sigma-Aldrich, HHS32, Sigma-Aldrich, HT110116) staining or immunohistochemistry (IHC; Dako, K5007). For IHC, the following antibodies were used: anti-MMP-1 (Abnova, PAB12708), anti-IL-8 (BIOSS, bs-0780R). The stained sections were analyzed under a Zeiss Axio Scope A1 microscope and Aperio CS2 slide scanner (Leica Biosystems).

Statistical analysis

All experiments were performed independently at least three times. In cases where one experimental group was compared with the control group, Student T-test was conducted under the assumption of normal distribution and homogenous variance. In the case of comparison of two or more experimental groups with the control group, normal distribution and homogeneity of variance were tested using the Shapiro-Wilk and Levene's tests, and appropriate statistical analysis was selected. In case where data were not normally distributed, non-parametric analysis (Kruskal-Wallis test) was conducted, and if the data were normally distributed, data were analyzed via analysis of variance (ANOVA) with post-hoc tests. At this time, the Bonferroni post-hoc test was conducted if the variance of the data group was homogenous, and Dunnett T3 post-hoc test was conducted if the variance was not homogeneous. All statistical analyses were conducted using SPSS Statistics v.25 (IBM). Significance was accepted at the 0.05 level of probability ($P < 0.05$).

Results

GLSO decreases the transcript levels of inflammatory factors during periodontal inflammation

To elucidate the effect of GLSO on periodontal inflammation, we initially examined the expression levels of

inflammatory factors under in vitro periodontal pathological conditions by treating cells with various doses of GLSO. PD-mimicking conditions were established by treating hGFs, the predominant cell type in periodontal tissue, with LPS, a crucial virulence factor. We observed that LPS-induced upregulation of well-known periodontal disease inflammatory factors, such as *MMP1* and *IL8*, was decreased by GLSO in a dose-dependent manner (Fig. 1A, B). Gingival inflammation is strongly associated with elevated levels of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α. These mediators can trigger a cascade of irreversible destruction of connective and bone tissues. In this study, IL-1β was used as a representative pro-inflammatory mediator during periodontal disease pathogenesis. GLSO significantly blocked the IL-1β-induced upregulation of *MMP1* and *IL8* in hGFs (Fig. 1C, D). Conversely, the expression of *IL6* and *PTGS2*, other well-known periodontal disease inflammatory factors, showed no significant changes even under periodontal pathological conditions treated with GLSO (Fig. 1A, C). Therefore, we focused on the anti-inflammatory effect of GLSO on periodontal inflammation using hGFs.

GLSO inhibits the functions of inflammatory proteins in pathogenic hGFs

Next, we investigated whether GLSO treatment also affected protein expression and activity in pathogenic hGFs. Consistent with the results shown in Fig. 1A, GLSO treatment inhibited the IL-1β-induced upregulation of inflammatory protein expression (Fig. 2A). Additionally, GLSO treatment significantly decreased the matrix-degrading activities of secreted MMPs in a dose-dependent manner, as evidenced by the total MMP activity assay under IL-1β treatment (Fig. 2B). This inhibitory effect of GLSO was further confirmed in

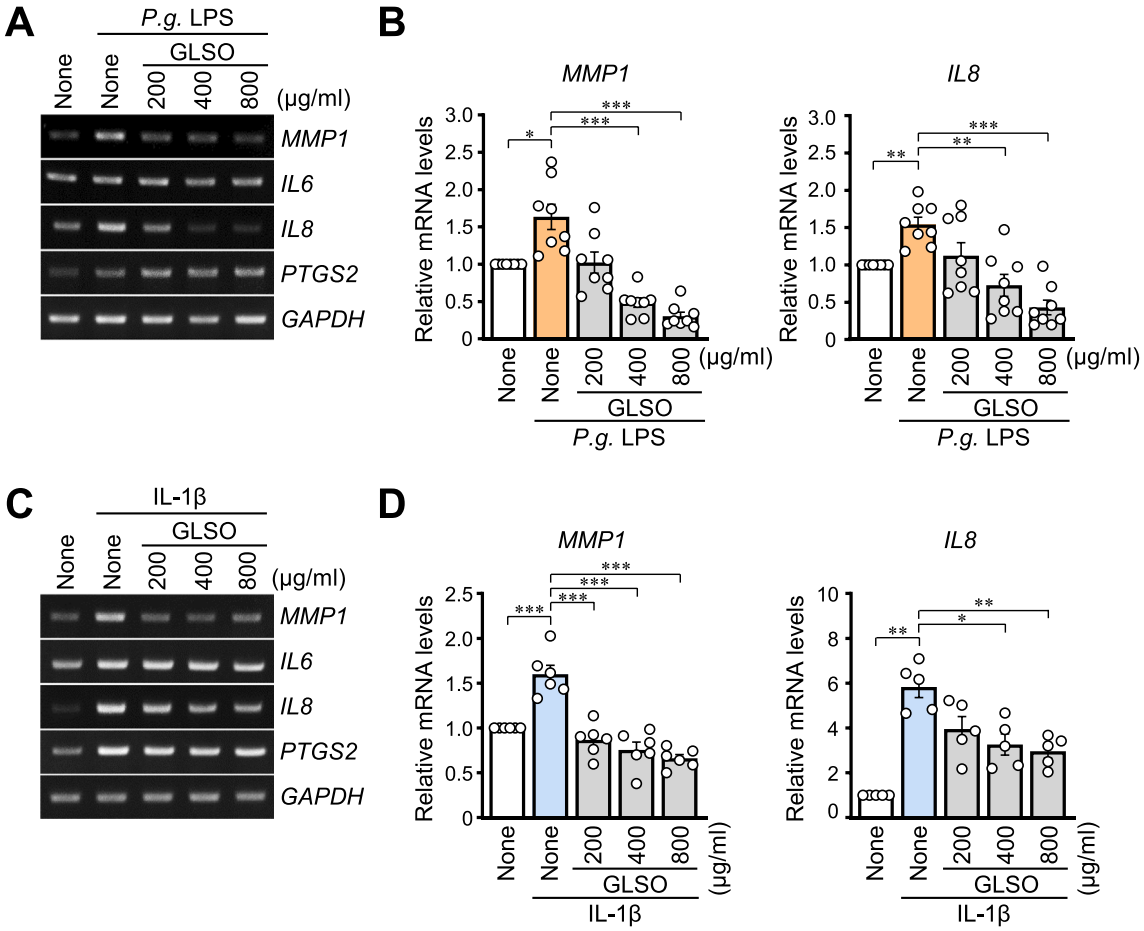

Fig. 1 GLSO suppresses mRNA expression of inflammatory factors in hGFs. **A**–**D** RT-PCR (**A** and **C**) and qRT-PCR (**B** and **D**) results of *MMP1*, *IL6*, *IL8*, *PTGS2*, and *GAPDH* mRNA expression levels in hGFs pre-treated with GLSO for 30 min before stimulation with 5 μg/ml P.g. LPS (**A** and **B**, n = 8) or 2 ng/ml IL-1β (**C** and **D**, n ≥ 5) for 24 h. Data are presented as mean ± SD. n indicates the number of biologically independent experiments. Statistical analyses were performed using one-way ANOVA followed by Games-Howell's post-hoc test (B, MMP-1 of D) or Tukey's post-hoc test (IL-8 of D). **P* < 0.05, ***P* < 0.01, ****P* < 0.001

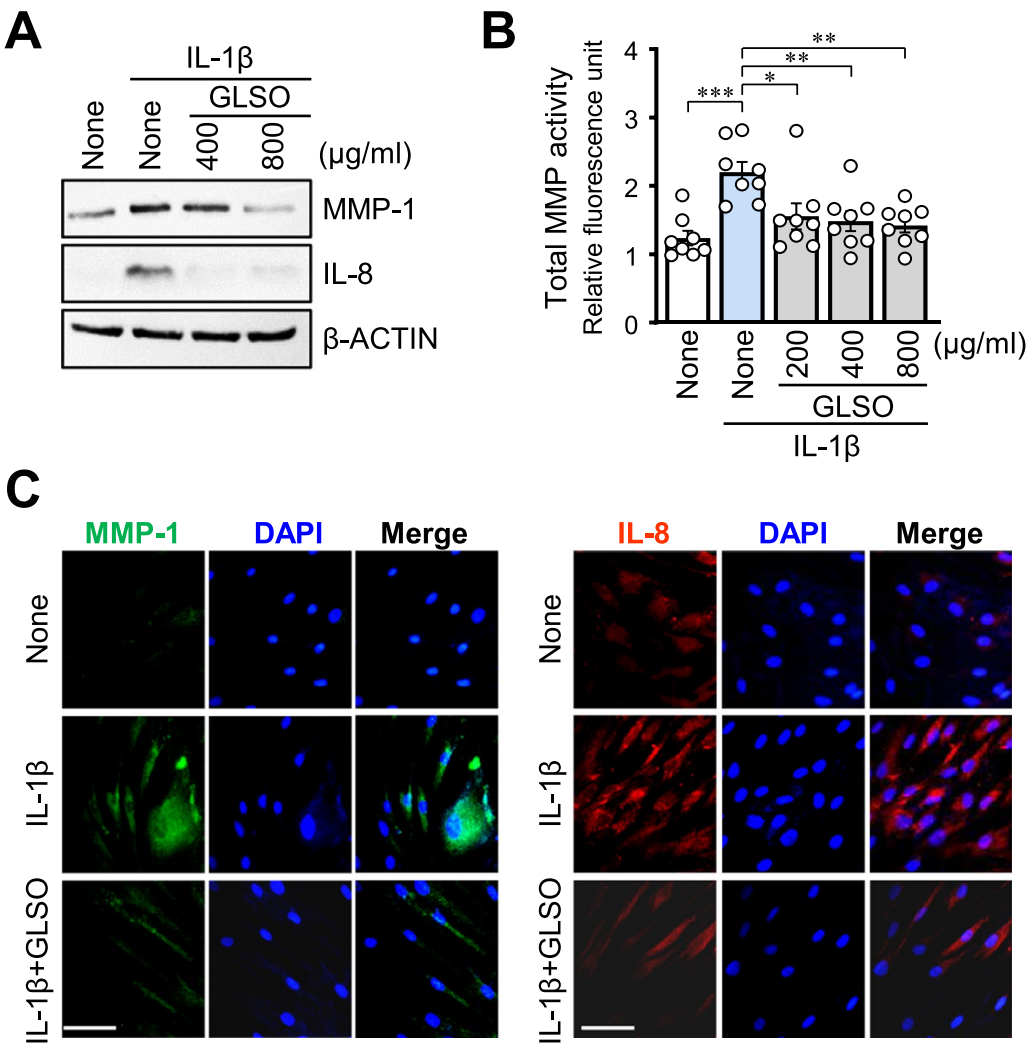

Fig. 2 GLSO suppresses the protein expression of inflammatory factors in hGFs. **A** Western blot results of MMP-1, IL-8, and β-ACTIN protein expression levels in hGFs pre-treated with GLSO for 30 min before stimulation with 2 ng/ml IL-1β for 24 h (n ≥ 3). **B** Relative fluorescence unit for total MMPs activity in cultured media of hGFs treated with GLSO in presence of 2 ng/ml IL-1β for 24 h (n = 8). **C** Representative immunofluorescence staining for MMP-1 (Green), IL-8 (Red), and DAPI (Blue) in hGFs treated with 2 ng/ml IL-1β or 2 ng/ml IL-1β and 800 μg/ml GLSO (n = 3). Scale bar, 50 μm. Data are presented as the mean ± SD. n indicates the number of biologically independent experiments. Statistical analysis was performed using Kruskal–Wallis test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

the experimental group treated with IL-1β, as shown by immunofluorescence assay images (Fig. 2C). In line with the changes in transcript levels, GLSO treatment suppressed the upregulation of both protein expression and activity of the examined inflammatory factors in pathogenic hGFs.

GLSO exerts its effects through the c-Jun N-terminal kinase (JNK) signaling pathway

To elucidate the mechanism behind GLSO's inhibitory effect on the expression of MMP-1 and IL-8 involved in periodontal inflammation, we investigated the activation of downstream signaling pathways previously reported to be relevant [32]. We measured the total protein expression and phosphorylation levels of three MAP kinases: extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun N-terminal kinase (JNK) in IL-1β-treated hGFs (Fig. 3A). Additionally, NF-κB signaling, indicated by the degradation of the inhibitor of NF-κB alpha (IκBα), was assessed using Western blotting. Figure 3A shows that the ERK and NF-κB signaling pathway peaked at 15 min after IL-1β stimulation, while NF-κB signaling was activated between 15 and 30 min. Next, we focused on the activation of ERK, JNK, and NF-κB by IL-1β treatment to determine the inhibitory effect of GLSO on IL-1β-induced gene expression. Co-treatment

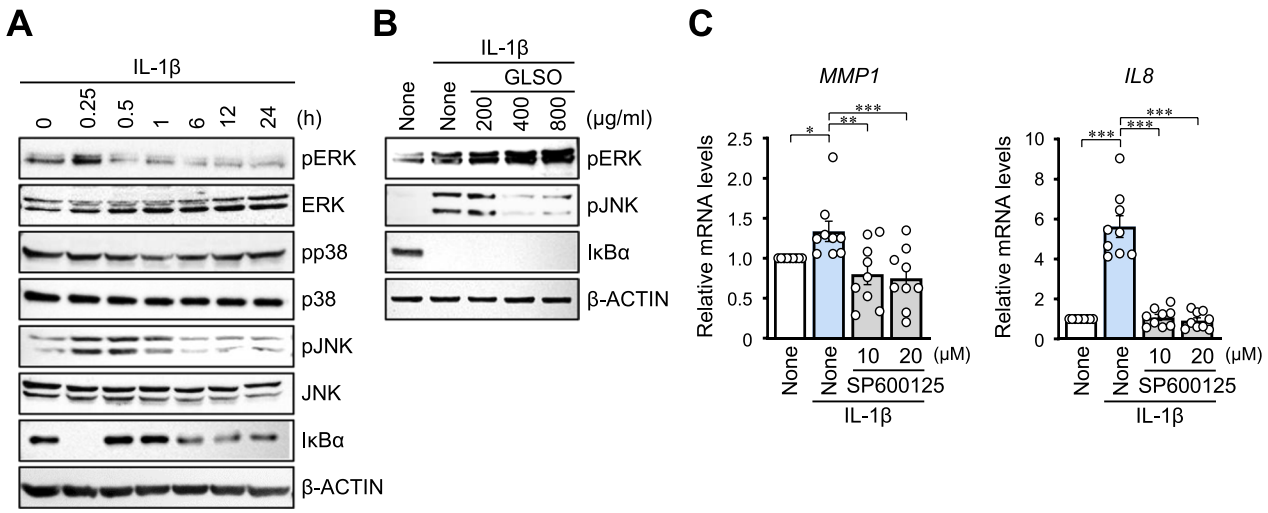

Fig. 3 GLSO regulates the expression of inflammatory factors by suppressing the JNK pathway activated by IL-1β in hGFs. **A** Western blot results of ERK, p38, JNK, and their phosphorylated forms (pERK, pp38, pJNK) and IκBα in hGFs treated with 2 ng/ml IL-1β for the indicated time (n ≥ 3). **B** Protein levels of pERK, pJNK and IκBα in hGFs treated with GLSO in presence of the 2 ng/ml IL-1β for 15 min (n ≥ 3). **C** qRT-PCR analysis of MMP1 and IL8 mRNA expression levels in hGFs treated with SP600125 (JNK inhibitor) in the presence of 2 ng/ml IL-1β for 24 h (n = 9). Data are presented as mean ± SD. n indicates the number of biologically independent experiments. Statistical analyses were performed using Kruskal–Wallis test (MMP-1 of C) and one-way ANOVA followed by Games-Howell's post-hoc test (IL-8 of C). **P* < 0.05, ***P* < 0.01, ****P* < 0.001

with GLSO in the presence of IL-1β significantly blocked IL-1β-induced activation of the JNK pathway but did not affect ERK and NF-κB signaling (Fig. 3B). This was validated by treatment with SP600125, a specific JNK inhibitor, which prevented the IL-1β-induced expression of MMP-1 and IL-8 in hGFs (Fig. 3C). Collectively, these data suggest that the inhibitory effects of GLSO on the upregulation of MMP-1 and IL-8 are mediated through the JNK signaling pathway activated by IL-1β.

GLSO ameliorates ligature-induced alveolar bone loss and periodontal inflammation in mice

To investigate the role of GLSO in periodontal disease (PD) in vivo, we administered GLSO via intraperitoneal injection into a ligature-induced PD mouse model. GLSO injection was initiated 1 day after ligature surgery and continued for 9 days [30]. The control group was injected with Polyethylene glycol-400 (PEG-400) via intraperitoneal injection [27]. Micro-computed tomography (μ-CT) results and histological analysis revealed a significant inhibition of alveolar bone loss in the experimental mouse PD model injected with GLSO (Fig. 4A, C). μ-CT scanning demonstrated a decrease in bone volume/total volume (BV/TV; %) (Fig. 4B) in the ligature-induced PD model, while intraperitoneal injection of GLSO resulted in an increase in BV/TV. A major phenotype of PD is the increase in alveolar bone loss, evaluated as the cementum-enamel junction (CEJ) to alveolar bone crest (ABC) distance measured via μ-CT or histological analysis. The CEJ-ABC distance was increased in the ligature-induced PD compared to the control group. Intraperitoneal injection of GLSO resulted in a decrease in alveolar bone loss, as evidenced by the decreased CEJ-ABC distance (Fig. 4D). These results demonstrate that alveolar bone loss occurs due to ligature surgery, and injection of GLSO alleviates BV/TV and CEJ-ABC distance. Next, we validated the in vivo catabolic role of GLSO in PD pathogenesis by detecting MMP-1 and IL-8. As shown in Fig. 5A and B, intraperitoneal injection of GLSO exhibited a significant reduction in periodontal inflammation and alveolar bone destruction in the ligature-induced PD model, with decreased expression levels of MMP-1 and IL-8 in inflamed gingiva. Taken together, these data suggest that GLSO has a protective effect on alveolar bone loss in the experimental mouse PD model.

Discussion

Understanding the molecular mechanisms that drive PD pathogenesis is crucial for developing effective therapeutic strategies to prevent or manage the disease. In recent years, there has been growing interest in exploring the therapeutic potential of natural compounds derived from medicinal plants in the management of PD. GLSO, a natural product derived from the spores of the medicinal mushroom Ganoderma lucidum, has emerged as a potential therapeutic agent for PD management. In this study, we propose the novel anti-inflammatory action of GLSO in periodontal tissues, suggesting its potential as a new therapeutic and adjunctive agent for periodontal diseases. According to our experimental findings, GLSO

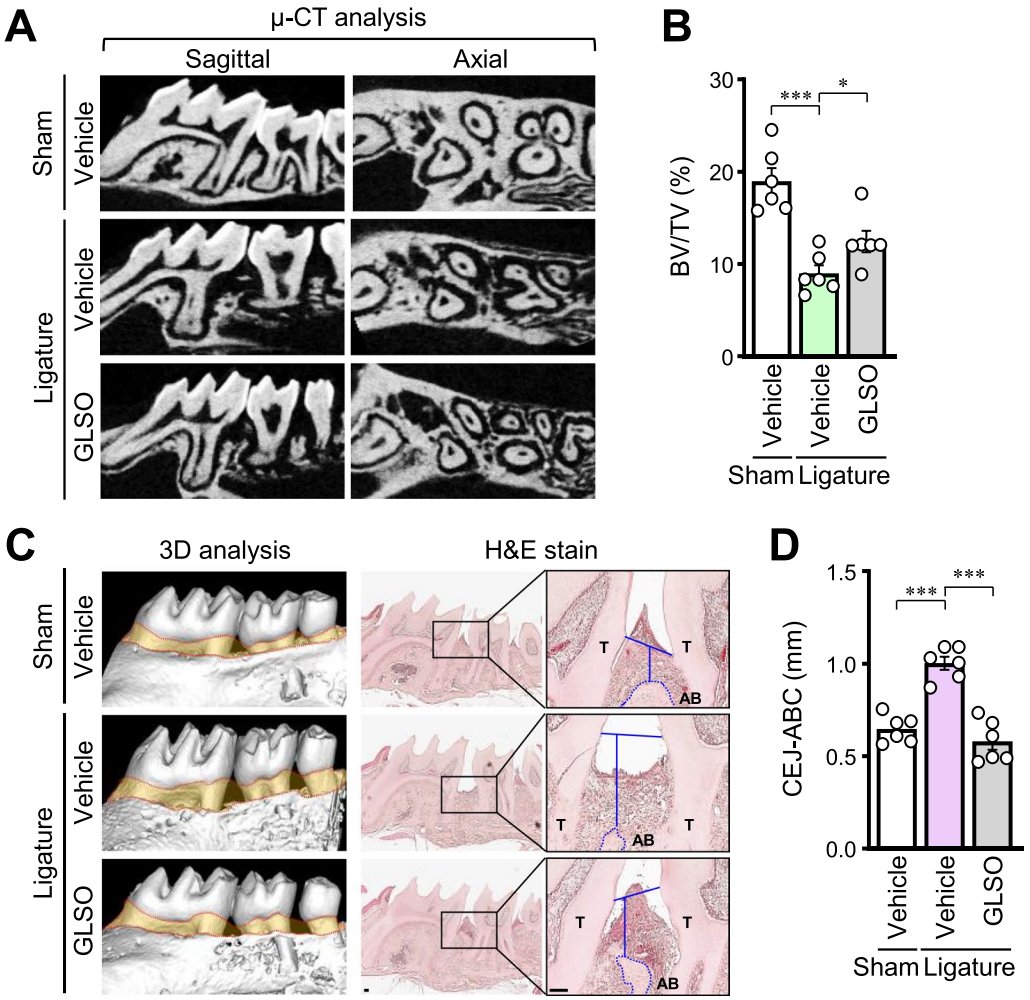

Fig. 4 Intraperitoneal injection of GLSO alleviates alveolar bone loss in mice with ligature-induced PD. **A** Representative μ-CT images of sagittal and axial-sectional views of maxillae from ligature-induced PD mice with IP injection of PEG-400 (vehicle) (n = 6) or GLSO (n = 6). **B** The percentage of BV/TV in the maxillae was measured via μ-CT reconstruction (n = 6). **C** Representative images of three-dimensional (3D) μ-CT and H&E staining of the maxillae from ligature-induced PD mice with IP injection of PEG-400 (vehicle) (n = 6) or GLSO (n = 6). Teeth regions were indicated by (T), and alveolar bone regions were indicated by dotted line (AB). The distance from the cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was marked by blue lines. Scale bar, 100 μm. **D** The distance from the CEJ to the ABC in the maxillae was measured via μ-CT reconstruction (n = 6). Data are presented as mean ± SD. n indicates the number of biologically independent experiments. Statistical analysis was performed using a two-tailed T-test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$

significantly attenuated the tissue inflammation and subsequent alveolar bone loss observed in a periodontitis mouse model. Through cell experiments using hGFs, this was inferred to be attributed to the suppression of increased expression of inflammatory factors MMP-1 and IL-8.

Periodontal disease is a multifactorial chronic inflammatory condition characterized by the uncontrolled inflammation of the periodontium and the tooth-supporting tissues [33]. The pathogenesis of PD involves a complex interplay of microbial dysbiosis, host immune response dysregulation, and environmental factors [34]. Infection by periodontopathic bacteria, such as *Porphyromonas gingivalis*, triggers a cascade of inflammatory responses, including the activation of Toll-like receptors by LPS, a component of the outer membrane of Gram-negative bacteria [35–37]. This activation leads to the elevated expression of inflammatory mediators, including IL-1β, IL-6, TNF-α, MMPs, and other proteases [38–40]. These inflammatory mediators contribute to tissue destruction and the progressive nature of PD. Our study unexpectedly found that GLSO reduced inflammatory factors induced by LPS and IL-1β but had no significant effect on TNF-α. IL-1β and TNF-α are pivotal cytokines

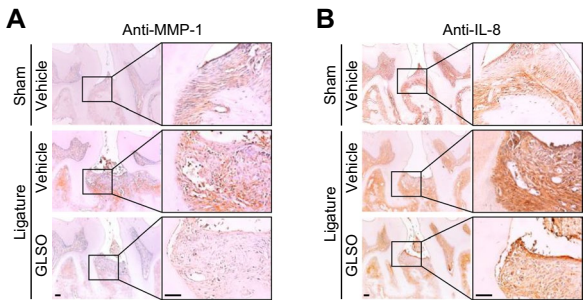

Fig. 5 Intraperitoneal injection of GLSO alleviates ligature-induced expression of MMP-1 and IL-8 in mouse gingival tissue. **A**, **B** Representative immunohistochemistry (IHC) staining for MMP-1 (**A**) and IL-8 (**B**) in gingiva from ligature-induced PD mice with IP injection of PEG-400 (vehicle) or GLSO (n = 4). Scale bar, 50 μm. n indicates the number of biologically independent experiments

in chronic inflammatory diseases, influencing diverse cellular activities such as proliferation, differentiation, and cell death. Despite sharing similarities, they differ in structure, cellular responses they elicit, and activation pathways [41]. This corresponds with prior research demonstrating varied responses to different inflammatory mediators. For instance, studies by Bonaventura [41] and Caramés [42] revealed the differential effects of these cytokines on metal metabolism control in rheumatoid arthritis and cell death in human articular chondrocytes, respectively. These findings suggest that GLSO may selectively modulate specific inflammatory pathways, underscoring the necessity for further investigation into its mechanism of action.

The gingival tissue comprises various cell types, including fibroblasts, epithelial cells, endothelial cells, and immune cells [43]. Among these, GF and periodontal ligament (PDL) cells predominantly inhabit the periodontal tissue and are known to overproduce inflammatory mediators such as nitric oxide and prostaglandin E2 (PGE2) during inflammatory processes [44]. Our study focused solely on GF due to their pivotal role in periodontal tissue homeostasis and disease progression. While this choice allowed for controlled experimentation, it's essential to recognize the limitation of not considering other cell types present in periodontal tissue, such as epithelial cells, endothelial cells, and immune cells. Future research should explore the contributions of these cell populations to gain a more comprehensive understanding of periodontal diseases and develop effective therapeutic strategies.

In our study, we observed that treatment with GLSO resulted in a significant reduction in the expression of inflammatory factors, including MMP-1 and IL-8, in hGFs subjected to inflammation. This reduction in inflammatory factors expression suggests that GLSO may exert anti-inflammatory effects by modulating the inflammatory response in periodontal tissues. Consistent with our findings, several studies have demonstrated the efficacy of GLSO in inhibiting inflammation and oxidative stress in various inflammatory conditions, including arthritis, colitis, dermatitis, and neuroinflammation [45–48]. It has been shown previously that GLSO significantly accelerated the process of skin wound healing by downregulation of the inflammation by regulating skin microbiota [45]. GLSO has been demonstrated to exhibit a diverse array of anti-cancer effects, including the inhibition of tumor growth [49]. One of the studies has demonstrated that this effect is caused by the induction of apoptosis through the activation of caspase-3 and -9 [18]. The anti-inflammatory and anti-tumor effects of GLSO are attributed to its bioactive constituents, such as triterpenoids, polysaccharides, and peptides, which modulate key signaling pathways involved in the inflammatory response. The core bioactive components of GLS encompass triterpenoids, polysaccharides, steroids, and fatty acids, with oleic acid and palmitate acid being predominant among the fatty acids [50]. Supercritical CO_2 extraction tends to yield higher levels of oleic acid [51]. Oleic acid, renowned for its efficacy in immunity, inflammation, cancer, and wound healing, may significantly contribute to the beneficial effects of GLSO. Heo et al. [27] also suggest that in a rheumatoid arthritis mouse model, the synergistic effects of fatty acids, particularly the abundance of oleic acid, may underpin the positive outcomes associated with GLSO. Additionally, Ganoderma lucidum has been traditionally employed in the prevention and treatment of various conditions, including liver diseases, high blood pressure, insomnia, and diabetes. Extracts from GLS have also been reported to exhibit bioactivities similar to those found in extracts from other parts [52]. Based on the various studies reviewed, it is deemed that the protective effects of GLSO in periodontitis (PD) observed in this study are sufficiently supported.

The principal signal transduction pathways implicated in periodontal inflammation include NF-κB, MAPK, and janus kinase-signal transducers and activators of transcription (JAK/STAT) pathways, collectively contributing to a multifaceted inflammatory network. NF-κB and MAPK signaling pathways are known to regulate inflammatory cytokines and MMP genes in periodontal diseases, particularly impacting gingival and alveolar bone tissues [53–55]. The MAPK family encompasses three main subfamilies—ERK-1/-2, JNK, and p38—whose cross-activation and interaction facilitate the integration of responses and the activation of distinct gene sets [56]. Previous studies have demonstrated that p38 activation induces the upregulation of various cytokines, including TNF-α, IL-8, IL-6, and PTGS2, through modulation

of both transcriptional and post-transcriptional mechanisms [57]. Moreover, inhibitors targeting JNK and ERK have shown efficacy in suppressing the production of pro-inflammatory mediators during the progression of periodontal disease [32]. In our presented results (Fig. 3A), treatment with IL-1β activated NF-κB and phosphorylated JNK (pJNK) in serum-free conditioned cultures of hGFs. GLSO treatment effectively inhibited the IL-1β-induced activation of the JNK pathway, while it did not significantly affect NF-κB activation triggered by IL-1β. These findings indicate that GLSO's protective effect against periodontal disease pathogenesis stems from its ability to inhibit MAP kinase pathways, rather than the NF-κB pathway. Other research groups have reported different molecular signaling mechanisms of GLSO. For instance, previous studies have shown that GLSO induces autophagy in colon cancer by inhibiting p38 MAP kinase and suppresses oxidative stress-induced invasive behavior in breast cancer cells by modulating ERK1/2 signaling [58]. Additionally, active lipids derived from GLSO treatment selectively enhanced the activation of JNK1/2 while reducing activation of ERK1/2 in human leukemia THP-1 cells [59]. Given the observed differences in downstream signaling triggered by various stimuli, further research is warranted to elucidate the underlying mechanisms.

Despite the compelling preclinical evidence supporting the anti-inflammatory attributes of GLSO, its therapeutic efficacy in managing periodontal disease remains largely unexplored. Our findings suggest that GLSO hold promise as natural therapeutic agents for PD prevention and treatment, particularly through the suppression of inflammation-mediated catabolic factors via inhibition of JNK signaling. Furthermore, given the reported antimicrobial effects of GLSO in other studies [52], its potential efficacy against periodontal pathogens, including *P. gingivalis*, is also anticipated. However, further research is needed to elucidate the underlying mechanisms of action and optimize treatment strategies for maximizing their therapeutic benefits in clinical settings. Additionally, clinical trials are warranted to evaluate the safety, efficacy, and long-term effects of GLSO in human subjects with PD.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-024-00941-3.

Additional file 1.

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Authors contribution
J.-H. K., and W.-Y. C. designed and performed most of the in vitro and in vivo studies. S.-J. J., M. K., and S.-C. J. participated in the animal studies and analyzed the data. S. K. and B.-J. C. performed RNA extraction and validated the data. G. L., and K. H. P. performed the statistical analysis and revised the manuscript. Y.-O. S. provided materials and conceived the project. J. -H. R. designed the study, wrote and revised the manuscript.

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Availability of data and materials
The presented data in this study are available on request from the corresponding author.

Declarations

Ethics approval and consent to participate
Prior to periodontal tissue collection, written informed consent was obtained from all participants, and the study protocol received approval from the Institutional Review Board of Chonnam National University Hospital (CNUDH-2023–010). Animal study was approved by the Animal Care and Ethics Committee of Chonnam National University (CNU IACUC-YB2023-96).

Consent for publication
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Competing interests
The authors declare no competing interests. Young-Ok Son is an Associate Editor of Applied Biological Chemistry.

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