Molecules and Cells

Avenanthramide-C Shows Potential to Alleviate Gingival Inflammation and Alveolar Bone Loss in Experimental Periodontitis

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Periodontal disease is a chronic inflammatory disease that leads to the gradual destruction of the supporting structures of the teeth including gums, periodontal ligaments, alveolar bone, and root cementum. Recently, interests in alleviating symptoms of periodontitis (PD) using natural compounds is increasing. Avenanthramide-C (Avn-C) is a polyphenol found only in oats. It is known to exhibit various biological properties. To date, the effect of Avn-C on PD pathogenesis has not been confirmed. Therefore, this study aimed to verify the protective effects of Avn-C on periodontal inflammation and subsequent alveolar bone erosion *in vitro* and *in vivo*. Upregulated expression of catabolic factors, such as matrix metalloproteinase 1 (MMP1), MMP3, interleukin (IL)-6, IL-8, and COX2 induced by lipopolysaccharide and proinflammatory cytokines, IL-1β, and tumor necrosis factor α (TNF-α), was dramatically decreased by Avn-C treatment in human gingival fibroblasts and periodontal ligament cells. Moreover, alveolar bone erosion in the ligature-induced PD mouse model was ameliorated by intra-gingival injection of Avn-C. Molecular mechanism studies revealed that the inhibitory effects of Avn-C on the upregulation of catabolic factors were mediated via ERK (extracellular signal-regulated kinase) and NF-κB pathway that was activated by IL-1β or p38 MAPK and JNK signaling that was activated by TNF-α, respectively. Based on this study, we recommend that Avn-C may be a new natural compound that can be applied to PD treatment.

Keywords: alveolar bone loss, avenanthramide-C, gingival fibroblasts, inflammation, periodontitis

INTRODUCTION

Periodontal disease, more commonly known as gum disease, is a chronic inflammatory disease caused by a bacterial infection in the oral cavity. Periodontal disease can be classified into gingivitis and periodontitis (PD) (Kumar, 2019). Gingivitis is a mild inflammation of the gingiva. In contrast, PD is a more severe and involves inflammation of the surrounding hard tissues, such as the alveolar bone, supporting the teeth (Highfield, 2009). If left untreated, gingivitis may progress to PD, leading to the destruction of the periodontium including the gingiva, periodontal ligament (PDL), cementum, and alveolar bone (Sedghi et al., 2021). Periodontal disease increases with age, with a reported prevalence rate of approximately 70.1% in adults, aged 65 years and older (Eke et al., 2012). PD is a primary cause of tooth loss in adults. Periodontal disease and

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its complications deteriorate the quality of life of patients and contribute significantly to social and economic burdens (Eke et al., 2015). Over the years, several studies have focused on the bi-directional relationship between periodontal disease and systemic disorders including diabetes mellitus, cardiovascular disease, and metabolic syndrome (Sedghi et al., 2021). These findings have led to mounting interest in periodontal disease.

The gingival tissue is composed of several different cell types, such as fibroblasts, epithelial cells, endothelial cells, and immune cells. Gingival fibroblasts (GF) and PDL cells, found in the periodontal tissue, can overproduce various inflammatory mediators, such as nitric oxide (NO) and prostaglandin E_2 (PGE_2), during the inflammatory process (Bãge et al., 2011). Current knowledge concerning the pathogenesis of PD suggests that sustained gingival inflammation by keystone microbial pathogens and inflammatory mediators are critical to periodontal disease progression. Gingival inflammation is highly associated with high 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 the connective and bone tissues (Pan et al., 2019). Importantly, matrix metalloproteinases (MMPs), the enzyme induced by a bacterial infection or the inflammatory response, are mainly responsible for periodontal tissue destruction. During periodontal inflammation, the inhibition of various inflammatory factors can prevent the progression of PD. Consequently, this observation may be applied to the treatment and prevention of PD. However, the use of anti-cytokine drugs and soluble cytokine blockers is associated with significant unwanted side effects, including hemorrhage and gastrointestinal problems (Watanabe et al., 2020). Traditional PD therapies with adjuvant administration of antibiotics, corticosteroids, and NSAIDs (non-steroidal anti-inflammatory drugs) also have limitations including microbial resistance, liver damage, and heart failure with long-term use (Pretzl et al., 2019). To effectively treat PD and minimize unwanted side effects, it is necessary to develop a naturally-derived alternative therapeutic agent that targets pro-inflammatory mediators.

Avenanthramides (Avns) are a unique type of polyphenolic alkaloid extracted from oat (*Avena sativa* L., *Poaceae*) that contain bioactive compounds, such as polyphenols (Xochitl et al., 2021). In several studies, natural and synthetic Avns have demonstrated potent anti-oxidant (Hernandez-Hernandez et al., 2021), anti-atherogenic (Thomas et al., 2018), anti-inflammatory (Kang et al., 2018), and anti-histamine (Dhakal et al., 2019) effects. Avn-A, -B, and -C are the major forms of Avns. Of these, Avn-C is the most abundant phytochemical compound in oat (Kang et al., 2018). Avn-C contributes to the inhibition of atherosclerosis through the inhibition of vascular smooth muscle cell proliferation and enhancement of eNOS (endothelial NOS) and NO expression and production (Nie et al., 2006). The most characteristic effect of Avn-C is its anti-inflammatory effect through the inhibition of nuclear factor kappa-light-chain-enhancer of the activated B cells (NF-κB). Moreover, Avn-C can also prevent pulmonary inflammation by inhibiting hypoxia-induced COX2 expression via SIRT1 activation in lung cancer cells (Lim and Kang, 2020). Previous studies have demonstrated that Avns or Avn-C have essential functions in several biological activities, including anti-oxidant (Hernandez-Hernandez et al., 2021), anti-cancer (Fu et al., 2019), anti-itch (Sur et al., 2008), and anti-inflammatory (Kang et al., 2018) functions. To date, the effect of Avn-C in periodontal disease has not been examined. Hence, we investigated the anti-inflammatory effects of Avn-C in human gingival cells and its protective role on alveolar bone erosion in a PD experimental mouse model.

MATERIALS AND METHODS

Isolation and culture of human GF and PDL cells

Gingival tissues were collected from interdental papillae of extracted teeth (extracted for orthodontic reasons) of 10 healthy donors. Gingival tissues were immediately washed approximately 10 times in PBS (phosphate-buffered saline) and then minced into small pieces. The gingival tissue pieces were plated in 100-mm culture dishes and incubated in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% penicillin, and 1% streptomycin. The PDL was isolated from the middle of third of the root and placed in 100-mm culture dishes in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Cells were expanded until passage 6 (P6) and used between P4 to P6 for the experiments. All patients provided written informed consent before tissue collection. The institutional review board of Chonnam National University Hospital reviewed and approved the study protocol (DTMP-2022-005).

Cell culture and Avn-C treatment

Human GF and PDL cells were stimulated with a concentrate of *Escherichia coli* lipopolysaccharide (LPS; Sigma-Aldrich, USA), human IL-1β (GenScript, USA), and human TNF-α (Enzynomics, Korea) in a serum-free medium for 24 h with or without a concentrate of Avn-C (36465; Sigma-Aldrich) dissolved in DMSO. For confirmation of the cellular pathways, the cells were treated with several inhibitors, including PD98059 (extracellular signal-regulated kinase [ERK] inhibitor), SB203580 (p38 mitogen-activated protein kinase [MAPK] inhibitor), SP600125 (c-Jun N-terminal kinase [JNK] inhibitor), Bay11-7082 (NF-κB inhibitor) with IL-1β, or TNF-α.

Cell viability

To assess cell viability, human GF or PDL cells were seeded into a 96-well dish at a density of 3×10^4 cells/well and treated with Avn-C (50, 100, and 200 μM). Post-incubation, the MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, and the cells were incubated for 2 h at 37°C in a CO_2 incubator. Next, the culture medium was removed, DMSO was added to each well and then the absorbance was measured at 562 nm.

RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNA was isolated using the TRIzol reagent and cDNA was obtained by reverse transcription. Quantitative PCR was performed using a StepOnePlus Real-time PCR system and SYBR premix Ex Taq (PR420; Takara Bio, Japan). For each target gene, the individual transcript levels were normalized to those of GAPDH and expressed as a fold change relative to

the indicated controls. Details relating to the primer used in the experiments are shown in Supplementary Table S1.

MMPs activity assays

Human GF were treated with the indicated concentrations of Avn-C in the presence of 1 μg/ml of LPS or 2 ng/ml of IL-1β or 50 ng/ml of TNF-α for 24 h. The cell culture supernatants were harvested and analyzed for MMP1 (F1M00; R&D Systems, USA) and MMP3 (ab118972; Abcam, UK) activity using a quenched fluorogenic-substrate-based enzyme activity assay kit according to the manufacturer's instructions. MMP1 activity was detected at Ex/Em = 320/405 nm. MMP3 activity was measured fluorescence at Ex/Em = 325/393 nm.

Western blot

Protein extracted from cells was isolated in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM NaF, 1% NP-40, 0.5% deoxycholate, 0.2% SDS) with a phosphatase and protease inhibitor cocktails (Roche, Switzerland) on ice for 30 min and centrifuged at 13,000 rpm for 30 min at 4°C. The supernatant was collected and the protein concentrations were determined. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blotted 5% skim milk for 1 h and incubated with primary antibodies at 4°C overnight. The followed antibodies were used : anti-MMP1 (NBP2-22123; Novus, USA), anti-MMP3 (ab52915; Abcam), anti-IL-6 (NB600-1131; Novus), anti-IL-8 (orb229133; Biorbyt, UK), anti-COX2 (aa 570-598; Cayman, USA), anti-pERK (#9101; Cell Signaling Technology, USA), anti-ERK (610408; BD Biosciences, USA), anti-pp38 (#9216; Cell Signaling Technology), anti-p38 (sc-535; Santa Cruz Biotechnology, USA), anti-pJNK (#9255; Cell Signaling Technology), anti-JNK (#9252; Cell Signaling Technology), anti-IκBα (sc-371; Santa Cruz Biotechnology), anti-β-ACTIN (A3854; Sigma-Aldrich). The blots were incubated with HRP-conjugated secondary antibody and detected using ECL solution (Cytiva) visualized by an EZ-capture MG system.

Ligature-induced PD mouse model

Experimental PD was induced in 10-week-old C57BL/6J male mice. Eight mice were used per experimental group. Briefly, 5-0 silk ligature was tied around the left second molar in the maxilla, and the right side was used as a control. Thereafter, 2 μl of 200 μM Avn-C or 0.1% DMSO was injected intra-gingivally between the first and the second molars using a syringe (32 GA, 9.25 mm, 30°, 7803-04; Hamilton, USA) once per day for a week. Mice were sacrificed 8 days after placement of the ligature. All animal procedures were approved and conducted as per the Animal Care and Ethics Committees of Chonnam National University (CNU IACUC-YB-2022-60).

Micro-computed tomography (μ-CT) analysis

μ-CT scanning of the maxilla was performed, as described in a previous study (Kim et al., 2017). Mice maxillae were fixed in 10% neutral buffered formalin and examined using the SkyScan 1172 μ-CT scanner. The x-ray source was set to 49 kV and 200 μA with a 0.5 mm aluminum filter at 11 μm resolution. μ-CT data were analyzed for alveolar bone loss between the first and the second molar regions in the buccal side after setting the ROI (region of interest) around the first and second molars, using CTAn (Bruker, USA) and Mimics 14.0 (Materialise, Belgium). The bone mineral density (BMD) and bone volume per tissue volume (BV/TV) were measured within the region of interest, which was set to the bone between the first and second molar. Bone loss was quantified by measuring the distance from the buccal cemento-enamel junction (CEJ) to the alveolar bone crest (ABC).

Histologic and immunohistochemistry staining

Mice maxillae were decalcified in 0.5 M EDTA (pH 7.4) for 2 weeks. After dehydration, the tissues were embedded in paraffin and sectioned at 5-μm thickness. The paraffin sections were stained with H&E or used for immunohistochemistry (IHC). For immunohistochemistry, sections were placed on slides and incubated with 0.1% trypsin for 40 min at 37°C after incubation in 3% H_2O_2 for 10 min. After blocking with 1% bovine serum albumin for 1 h, slides were incubated with the following antibodies, anti-IL-6 (NB600-1131; Novus) and anti-IL-8 (orb229133; Biorbyt) followed by staining using the EnVision HRP (K5007; Dako, Denmark) and AEC substrate kit (SK-4200; Vector Laboratories, USA). Sections were counterstained with Hematoxylin (S3309; Dako). Osteoclasts on the alveolar bone surface were detected from the section using tartrate-resistant acid phosphatase (TRAP) staining. After deparaffinization and rehydration, tissues were stained with TRAP staining solution (PMC-AK04F; Cosmo Bio, Japan) for 30 min in 37°C incubator. For counterstaining, 0.08% fast green (F7258; Sigma-Aldrich) was used.

Statistical analysis

All experiments were repeated at least three times. Values are presented as mean ± SD. Statistical analyses were performed using Prism 7 (GraphPad Software, USA). Comparisons among 3 or more groups were carried out using one-way ANOVA followed by Tukey's post hoc test (multi-comparison). The n value represents the number of independent experiments or mice. Results were considered statistically significant at P values < 0.05.

RESULTS

Avn-C decreased the transcript levels of catabolic factors during periodontal inflammation

To elucidate the effect of Avn-C on periodontal inflammation, we initially examined the expression level of inflammatory factors under *in vitro* periodontal pathological conditions by treating the cells with various doses of Avn-C. PD-mimicking conditions were produced by treating the human GF and PDL cells (the predominant cell types in periodontal tissue) with a crucial virulence factor, LPS, or representative pro-inflammatory cytokines including IL-1β and TNF-α. Initial validation via the MTT assay demonstrated that Avn-C treatment had no impact on cell viability (data not shown). We observed that LPS-induced upregulation of well-known PD inflammatory factors, such as *MMP1, MMP3, IL-6, IL-8*, and *COX2* in human GF, were decreased by Avn-C in a dose-dependent manner (Fig. 1A). In the present investigation, *E. coli*-derived LPS was chosen over *Porphyromonas gingivalis*-derived LPS due

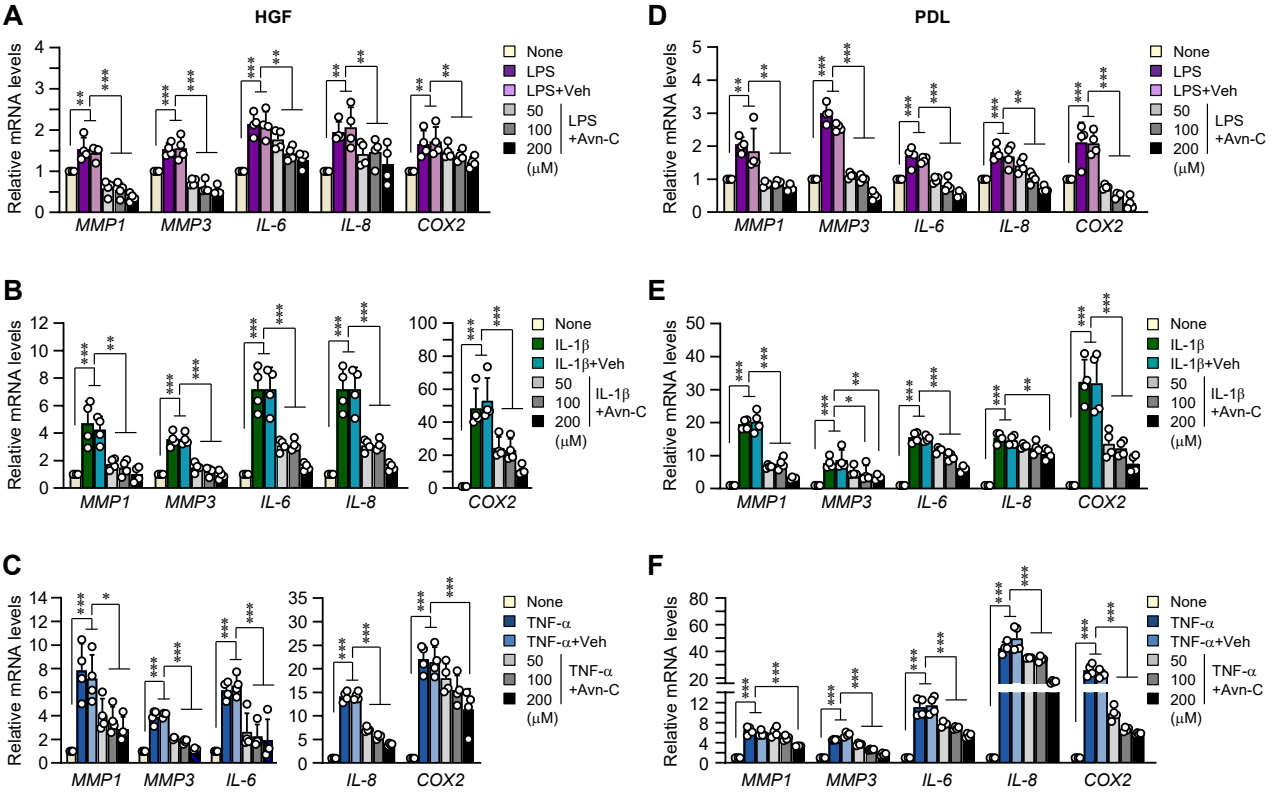

Fig. 1. Avn-C inhibits pathological condition-induced inflammatory factors expression in both human GF and PDL cells. (A-C) Quantitative real time-PCR analysis of transcript levels treated with the indicated concentration of Avn-C in the presence of 1 μg/ml *Escherichia coli* LPS (A) or 2 ng/ml IL-1β (B) or 50 ng/ml TNF-α (C) for 24 h in human GF (n = 4). (D-F) mRNA levels in PDL cells treated with indicated concentration of Avn-C in the presence of 1 μg/ml *E. coli* LPS (D) or 2 ng/ml IL-1β (E) or 50 ng/ml TNF-α (F) for 24 h (n = 4). Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. All experiments were repeated at least three times. Avn-C, Avenanthramide-C; GF, gingival fibroblasts; PDL, periodontal ligament; HGF, human gingival fibroblasts; LPS, lipopolysaccharide; Veh, vehicle.

to its demonstrated propensity, in line with previous literature (Jain and Darveau, 2010; Liu et al., 2008), for inducing a more robust upregulation of inflammatory factor. In addition, Avn-C significantly blocked the IL-1β (Fig. 1B) or TNF-α (Fig. 1C)-induced upregulation of *MMP1, MMP3, IL-6, IL-8*, and *COX2* in human GF. Consistent with this, Avn-C in PDL cells showed significant protective effects in periodontal pathological conditions such as LPS (Fig. 1D), IL-1β (Fig. 1E), or TNF-α (Fig. 1F). GF are one of the most abundant cells in gingival tissue and are firstly exposed to pathogens at the onset stage of PD (Naruishi, 2022). Therefore, we focused on the anti-inflammatory effect of Avn-C on periodontal inflammation using human GF.

Avn-C inhibited the protein functions of inflammatory factors in pathogenic human GF

Next, we verified whether the protein expression and functions were also altered by Avn-C in pathogenic human GF. Consistent with the results shown in Fig.1A, Avn-C treatment inhibited the upregulated protein expression of the inflammatory factors by LPS (Fig. 2A). In addition, matrix-degrading enzymatic activities due to secreted MMP1 and MMP3 were significantly decreased by Avn-C in a dose-dependent manner (Fig. 2B). The inhibitory effect of Avn-C was also shown in the experimental group treated with IL-1β (Figs. 2C and 2D) and TNF-α (Figs. 2E and 2F). Consistent with the change of transcript levels, upregulation of protein expression and functions of the examined catabolic factors in pathogenic human GF was suppressed by Avn-C treatment.

Avn-C inhibited IL-1β-induced ERK and NF-κB activation

The severity of inflammation and alveolar bone loss associated with LPS-induced periodontal disease is mostly dependent on upregulation of pro-inflammatory cytokines such as IL-1β and TNF-α (Zhou et al., 2021). To address the mechanism of the inhibitory effect of Avn-C on the increased expression of catabolic factors by pro-inflammatory cytokines, we focused on the activation of the related downstream signaling pathways based on previous studies (Tran et al., 2021). The total protein expression and phosphorylation level of three MAP kinases, namely, the ERK, p38 MAPK, and JNK, were determined in IL-1β-treated human GF. NF-κB signaling was determined by the degradation of the inhibitor of NF-kB alpha (IκBα) via Western blotting. Figure 3A shows that the ERK pathway peaked at 15 min after IL-1β stimulation, and the NF-κB signaling was activated in 15-30 min. In the next experiment, it was confirmed whether the activated signaling pathways identified in the above results were regulated by

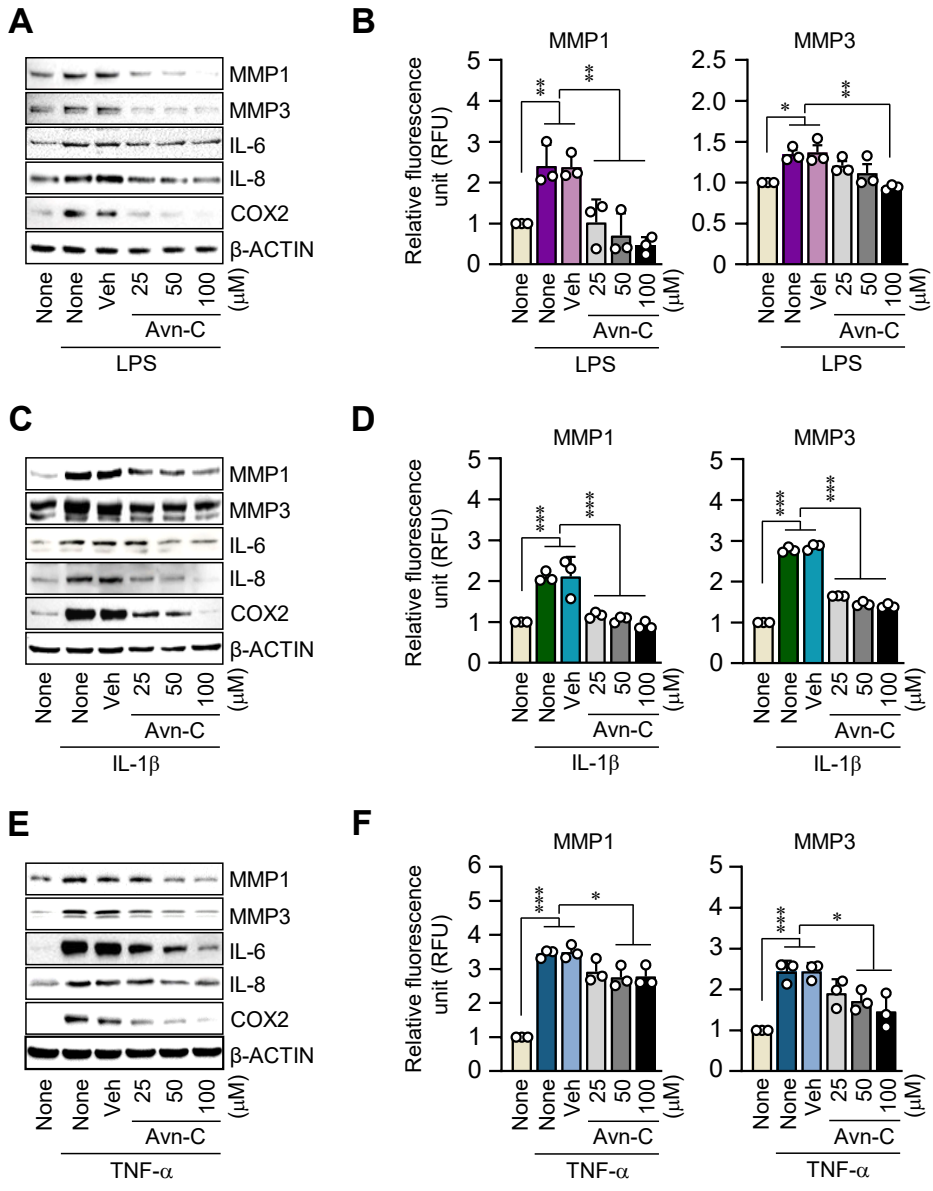

Fig. 2. Avn-C suppresses the expression of the inflammatory factors and activities of MMPs during PD-like pathogenesis *in vitro* in human GF. (A, C, and E) Protein levels in human GF treated with indicated concentration of Avn-C in the presence of 1 μg/ml *Escherichia coli* LPS (A) or 2 ng/ml IL-1β (C) or 50 ng/ml TNF-α (E) for 24 h (n = 3). (B, D, and F) The MMP1 and MMP3 activities in human GF treated with Avn-C in the presence of LPS (B) or IL-1β (D) or TNF-α (F) (n = 3). Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. Avn-C, Avenanthramide-C; MMPs, matrix metalloproteinases; PD, periodontitis; GF, gingival fibroblasts; Veh, vehicle; LPS, lipopolysaccharide.

Avn-C treatment. Co-treatment with Avn-C in the presence of IL-1β blocked the IL-1β-triggered activation of not only the ERK pathway but also NF-κB signaling (Fig. 3B). Treatment with PD98059, a specific inhibitor of phosphorylation in ERK (pERK), blocked the IL-1β-activated expression of *MMP1, MMP3, IL-6, IL-8,* and *COX2* (Fig. 3C). Additionally, Bay11-7082, a specific inhibitor of NF-κB signaling, also altered the expression of the catabolic mediators activated by IL-1β (Fig. 3D). These data suggest that the inhibitory effects of Avn-C on the upregulation of catabolic factors by IL-1β were mediated via blocking the ERK pathway and NF-κB signaling.

Avn-C inhibited TNF-α-induced p38 MAPK and JNK signaling pathways

Next, we investigated the molecular mechanism of the anti-inflammatory effects of Avn-C on the expression of inflammatory mediators stimulated by TNF-α. Inconsistent with the results from the IL-1β stimulation, TNF-α triggered p38 MAPK, JNK, and NF-kB activation determined by an increase of pp38 and pJNK or a decrease of IκBα expression in human GF, respectively. In contrast, ERK phosphorylation was not altered in the TNF-α-stimulated human GF (Fig. 4A). Compared with cells treated with TNF-α alone, the levels of pp38 and pJNK significantly decreased in TNF-α-stimulated cells treated with Avn-C in a dose-dependent manner (Fig. 4B). However, the degradation of IκBα did not alter by Avn-C treatment (Fig. 4B). The blockade of p38 by SB203580 (Fig. 4C) and JNK by SP600125 (Fig. 4D) inhibited the TNF-α-induced inflammatory gene expression. Together, these data suggest that Avn-C inhibits the catabolic effects of pro-inflammatory cytokines during PD pathogenesis by modulating different signaling pathways. We found that the inhibitory effects of Avn-C on the upregulation of catabolic factors were mediated via the ERK and NF-κB pathways activated by IL-1β, or p38 MAPK

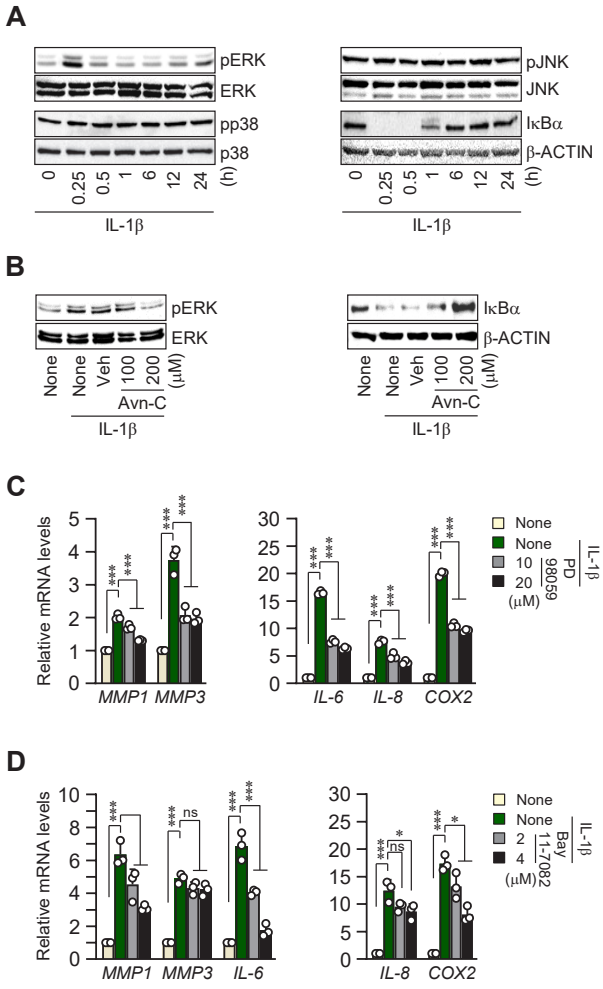

Fig. 3. Avn-C blocks IL-1β signaling by inhibiting the ERK and NF-κB pathways. (A) Human GF were treated with 2 ng/ml IL-1β for the indicated time. Phosphorylation of ERK, p38 MAPK, JNK, and IκBα, was determined by Western blot. (B) Protein levels in human GF treated with Avn-C in the presence of the 2 ng/ml IL-1β. (C and D) Quantitative real time-PCR of the indicated genes in human GF treated with 2 ng/ml IL-1β in the absence or presence of the selective ERK inhibitor PD98059 (C) or the NF-κB inhibitor Bay11-7082 (D) for 24 h (n = 3). Data are presented as mean ± SD. ns, not significant. $*P < 0.05$, $***P < 0.005$. Avn-C, Avenanthramide-C; GF, gingival fibroblasts; PD, periodontitis.

and JNK signaling activated by TNF-α, respectively.

Avn-C ameliorated ligature-induced alveolar bone loss in mice

To explore the role of Avn-C in PD *in vivo*, we administered Avn-C to the ligature-induced PD mouse model via intra-gingival injection. The major phenotypes of PD include a decrease in BMD and an increase in alveolar bone loss. The alveolar bone loss was evaluated as the CEJ to ABC distance measured via μ-CT or histological analysis. μ-CT scanning (Fig. 5A) revealed that BMD and BV/TV (%) were decreased, whereas CEJ-ABC distance was increased in ligature-induced

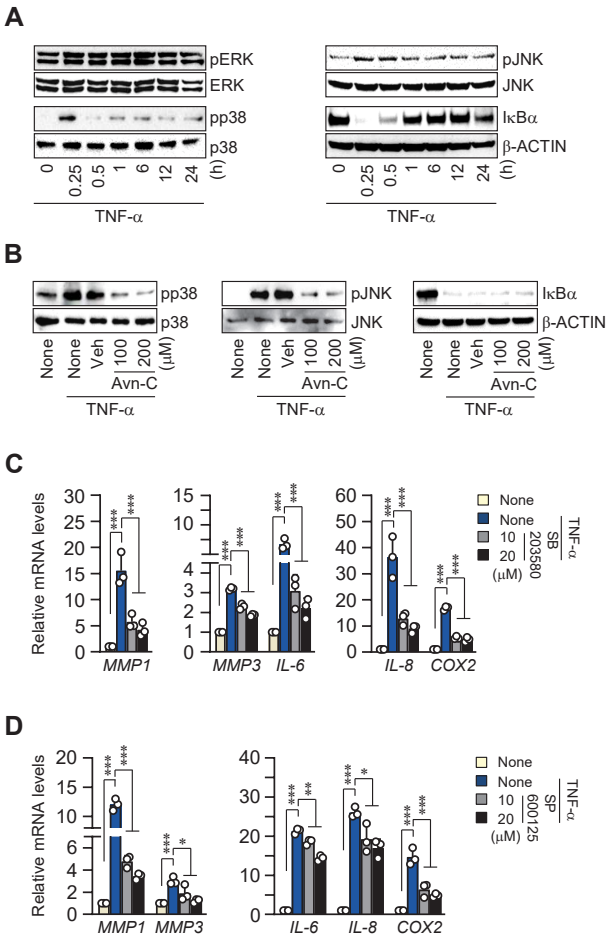

Fig. 4. Avn-C blocks TNF-α signaling by inhibiting the p38 MAPK, JNK, and NF-κB pathway. (A) Human GF were treated with 50 ng/ml TNF-α for the indicated time. (B) Protein levels in human GF treated with Avn-C in the presence of the 50 ng/ml TNF-α. (C and D) Quantitative real time-PCR of the indicated genes in human GF treated with 50 ng/ml TNF-α in the absence or presence of the selective p38 kinase inhibitor, SB203580 (C) or the JNK inhibitor, SP600125 (D) for 24 h (n = 3). Data are presented as mean ± SD. $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$. Avn-C, Avenanthramide-C; GF, gingival fibroblasts.

PD compared with the control group. Intra-gingival injection of Avn-C resulted in an increase of BMD (Fig. 5B) and BV/TV (Fig. 5C) and a decrease of alveolar bone loss, as evident from the decreased CEJ-ABC distance measured (Fig. 5D). Consistent with μ-CT results, the histological analysis showed significant inhibition of alveolar bone loss in the experimental mouse PD model after Avn-C injection (Fig. 5E). A decrease in the number of osteoclasts by Avn-C administration was also observed in TRAP staining results (Fig. 5F). Furthermore, we validated the *in vivo* catabolic role of Avn-C in PD pathogenesis by detecting IL-6 and IL-8. As shown in Figs.5G and 5H, intra-gingival injection of Avn-C led to a significant reduction of periodontal inflammation and alveolar bone destruction in the ligature-induced PD mouse model. There was also a decreased expression of IL-6 and IL-8 in the inflamed

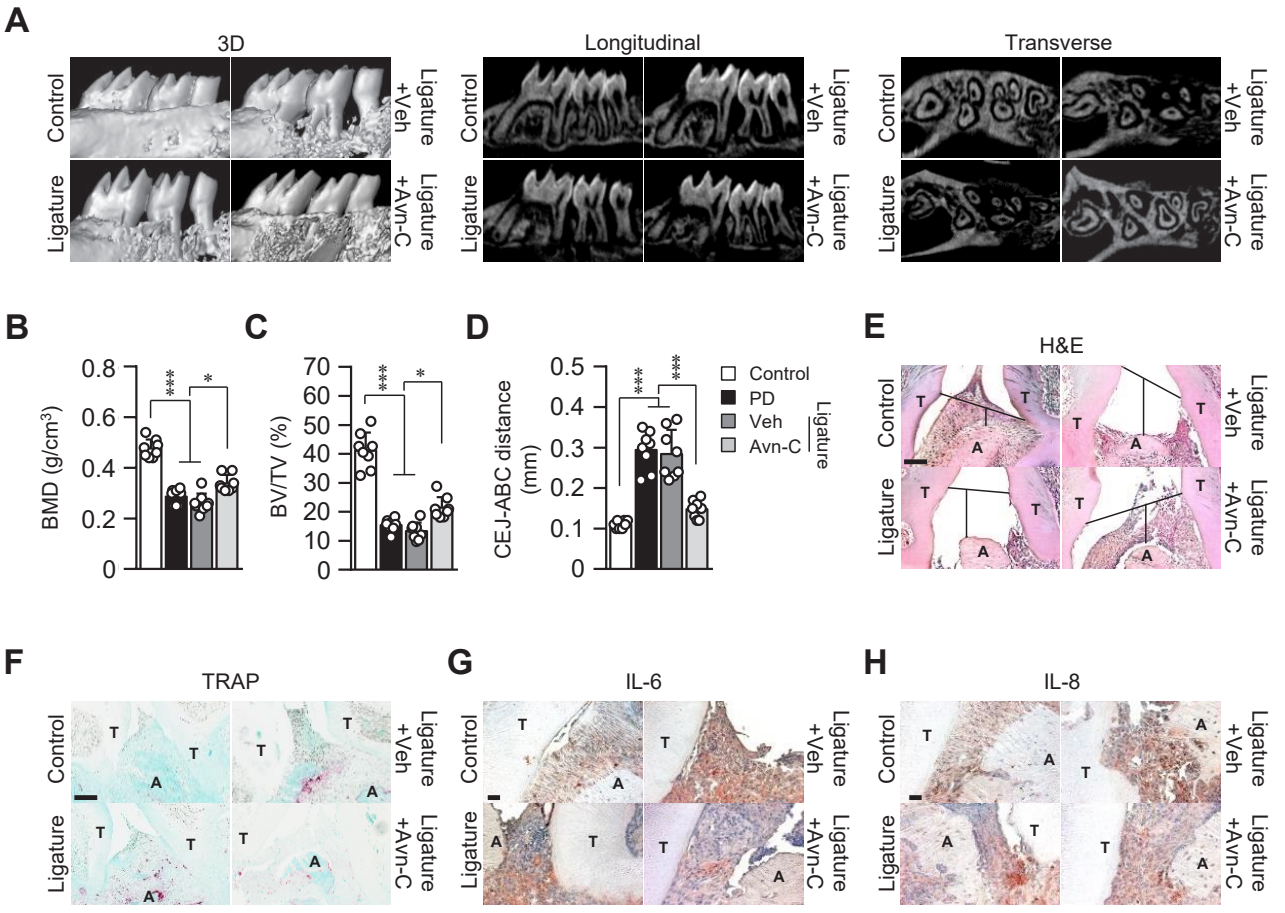

Fig. 5. The therapeutic effects of Avn-C on alveolar bone loss in ligature-induced PD in mice. Maxillary samples were divided into four groups (n = 8): Control (non-ligature), PD (ligature), PD + Veh (ligature + DMSO), and PD + Avn-C. (A) Representative μ-CT images of view of three-dimensional (3D), longitudinal, and transverse-sectional of the maxillae. BMD (B) and BV/TV (C) were measured from the bone between the first and second molars. (D) The distance of the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) was analyzed. (E) Representative images of H&E staining. Magnification, ×200. Scale bar = 200 μm. (F) Representative images of TRAP staining. Magnification, ×200. Scale bar = 200 μm. (G and H) Immunohistochemistry (IHC) staining for IL-6 (G) and IL-8 (H). Magnification, ×400. Scale bar = 50 μm. Data are presented as mean ± SD. $*P < 0.05$, $***P < 0.005$. Avn-C, Avenanthramide-C; PD, periodontitis; Veh, vehicle; μ-CT, micro-computed tomography; BMD, bone mineral density; BV/TV, bone volume/tissue volume; CEJ-ABC, cementum-enamel junction-alveolar bone crest; PD, periodontitis; T, tooth; A, alveolar bone.

gingiva. Taken together, these data suggest that Avn-C exerts a protective effect on alveolar bone loss in the experimental PD mouse model.

DISCUSSION

PD, the most common chronic inflammatory disease, is characterized by the uncontrolled inflammation of the periodontium. Infection by periodontopathic bacteria, such as *P. gingivalis* and their metabolites can trigger the elevated expression of inflammatory mediators including IL-1β, IL-6, TNF-α, MMPs, and other proteases (Kang et al., 2017; Zhang et al., 2022; Zhou et al., 2021). Understanding the molecular mechanisms that contribute to PD pathogenesis has led to the development of therapeutics that aimed to treat or manage the disease. Conventional treatment options for periodontal disease include anti-infective non-surgical treatment, such as the use of antibiotics to target pathogenic microorganisms and other prominent risk factors. However, the current therapy has several limitations, such as significant side effects from long-term use (Pretzl et al., 2019). Therefore, there is an urgent need to develop alternative therapeutics, especially natural agents, for PD treatment. Based on the results of this study, we recommend the use of Avn-C, derived from oats, as a promising therapeutic agent for PD prevention and treatment.

Dysbiosis of oral microorganisms is the primary etiologic factor associated with gingivitis and PD. Several risk factors are known to play an important role in an individual's response to periodontal infection, thus elucidation of the way to block these risk factors can help to target patients for prevention and treatment. This study proposed that Avn-C could

potentially inhibit inflammatory reactions within the periodontal tissues, mitigating the extent of periodontal inflammation and loss of alveolar bone density. To confirm whether Avn-C regulated the increase in susceptibility to oral microorganisms, the expression of toll-like receptor (TLR)2 and TLR4, receptors of bacteria, was additionally determined. Our supplementary experiments revealed that Avn-C did not regulated cytokine-induced increase of TLRs expression (data not shown). During inflammation, human GF and PDL cells have a pivotal role in the production of cytokines and chemokines. The regulation of inflammatory factors generated in those cells can be considered as an important treatment strategy for PD (Morandini et al., 2011). Therefore, understanding the cellular signaling processes involved in the inflammatory response in periodontal disease is essential for the development of new therapies that aim to prevent or attenuate periodontal destruction. GF and PDL cells are functionally different cell types in the periodontium. They both participate in the host immune response in PD. GF are the most abundant cell types in periodontal connective tissues. GF are exposed to pathogens at an early stage of infection. They are critical in sustaining inflammatory responses in periodontal disease. GF also have distinct functions in the repair of periodontal tissues. In contrast, protein and collagen production is significantly higher in PDL cells than that in GF (Banlue et al., 2023; Hudson et al., 2017; Somerman et al., 1988). PDL cells also have higher alkaline phosphatase activity when compared with those of GF (Garna et al., 2022). In the present study, we provided evidence that Avn-C exhibited significant anti-inflammatory activities in a dose-dependent manner both in human GF and PDL cells (Fig. 1). Nevertheless, in this study, we focused on the signaling pathways involving human GF, not PDL cells, after stimulation with pro-inflammatory cytokines. By focusing on human GF, which are located in the gingival connective tissue in close proximity to PDL cells, our research sheds light on the response of cells directly associated with gingival tissue. As periodontal diseases primarily affect the gingival tissue, studying the effects of Avn-C on human GF can have direct clinical implications. It allows us to understand the potential therapeutic benefits of Avn-C in managing and treating gingival inflammation, which is a common problem in periodontal diseases. However, additional research is needed to investigate the anti-inflammatory effects of Avn-C on PDL cells. While our study focused on human GF in gingival tissue, understanding the specific effects of Avn-C on PDL cells would provide a more comprehensive understanding of its therapeutic potential in managing periodontal inflammation.

The representative signal transduction pathways related to the inflammation of PD are NF-κB, MAPK, janus kinase-signal transducers, and activators of transcription (JAK/STAT) pathways and these contribute to a complex inflammatory network that influences the progression of periodontal disease (Souza et al., 2012). It is known that the NF-κB and MAPK signaling pathways are associated with the regulation of inflammatory cytokines and MMP genes in periodontal disease. In particular, NF-κB is detected in various signaling targets that correlate with inflammatory processes affecting the gingiva and alveolar bone supporting the teeth (Guan et al., 2022; Hong et al., 2022; Song et al., 2021). The MAPK family consists of three main subfamilies, namely, ERK-1/-2, JNK, and p38. The cross-activation and cross-interaction between the different MAPK subfamilies in the cascade may serve to integrate the responses and activate a separate set of genes. A previous study reported that p38 MAPK activation can lead to an increased expression of various cytokines, such as TNF-α, IL-8 IL-6, and COX2 by modulating both transcriptional and post-transcriptional regulation pathways (Tomida et al., 2015). In addition, inhibitors of JNK signaling and the ERK pathway have also shown efficacy in inhibiting the production of pro-inflammatory mediators during periodontal disease progression. In our results presented in Figs. 3 and 4, the comparison between IL-1β and TNF-α revealed important differences in the activation of these signaling pathways. NF-κB and pERK activated by IL-1β acted on the expression of catabolic factors. In contrast, NF-κB, JNK, and p38 MAPK were activated by TNF-α, and those signaling pathways acted on catabolic factor expression. Avn-C blocked the ERK and NF-κB pathways stimulated by IL-1β, and inhibited p38 MAPK and JNK signaling induced by TNF-α. Since the cause of discrepancies in the downstream signaling triggered by different stimuli cannot be identified as yet, further research is warranted.

Meanwhile, several studies have demonstrated the effectiveness of many natural compounds for treating periodontal disease. Natural polyphenols, which are abundant in fruits, especially berries, and vegetables, show excellent anti-oxidant, anti-inflammatory, anti-bacterial, immune regulation, and anti-cancer effects (Jiang et al., 2021). Other studies have reported that Avns exhibit anti-oxidant (Hernandez-Hernandez et al., 2021), anti-cancer (Fu et al., 2019), and anti-inflammatory effects (Kang et al., 2018) by inhibiting NF-κB activation in various cell types. Recently, studies have also demonstrated that Avn-C can restore Alzheimer's disease-associated memory and behavioral disorders (Ramasamy et al., 2020). Moreover, our previous study proposed that Avn-C is an effective candidate for preventing osteoarthritis progression (Tran et al., 2021). It has been reported that Avn-C can inhibit the NF-κB pathway through allosteric binding to IKKβ and subsequent downregulation of COX-2/PGE_2 pathway may represent anti-inflammatory effect of Avn-C in muscle cells (Kang et al., 2018). In addition, many studies have demonstrated that Avns can reduce the production of pro-inflammatory cytokines such as IL-6, IL-8, and MCP-1 by inhibiting NF-κB activation that is responsible for activating the genes of inflammatory response (Sur et al., 2008). Avn-C also exhibits anti-inflammatory effects by inhibiting the expression of pro-inflammatory cytokines like IL-6 and TNF-α, and MMPs through reduced MAPK/NF-κB activity in endothelial cells (Guo et al., 2008), arterial smooth-muscle cells (Park et al., 2021), and chondrocytes (Tran et al., 2021). Thus, these oat polyphenols mediate the decrease of inflammation by inhibiting the cytokine release. In current study, direct binding of Avn-C to NF-κB or MAPK signaling molecules or direct modulation of cytokine release are possible mechanisms explaining the anti-inflammatory effects of Avn-C on PD pathogenesis, and this issue remains to be further elucidated.

From the results of this study, we recommend that Avn-C can be used to inhibit PD progression. Our studies have

elucidated the inhibitory roles of Avn-C in both periodontal inflammation and the subsequent alveolar bone destruction in periodontal disease. Although the exact molecular mechanism pathway depends on the stimulus, we verified that the protective effect of Avn-C on PD pathogenesis is related to inhibiting the expression of inflammatory catabolic factors. Avn-C has the potential to alleviate PD progression and may be a good therapeutic agent for the management of periodontal disease.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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AUTHOR CONTRIBUTIONS

S.-J.K., S.H.L., and B.D.Q. carried out all experiments. T.-T. T., J.K., Y.-G.K., and W.-Y.C. analyzed data. S.Y.L. wrote and revised the manuscript. J.-H.R. designed the study and reviewed the manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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