Effects of aged garlic extract on experimental periodontitis in mice

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Received November 8, 2024; Accepted March 18, 2025

DOI: 10.3892/br.2025.1975

Abstract. Aged garlic extract (AGE) has been reported to exert anti-inflammatory effects. AGE has been recently found to reduce the inflammatory symptoms of periodontitis, a widespread chronic inflammatory disease caused by oral bacterial infection. However, the mechanisms underlying these effects remain unclear. In the present study, it was aimed to determine the effects of AGE on experimental periodontitis and the related inflammatory factors. AGE (2 g/kg/day) was orally administered to 15 mice during the experimental period, while a control group consisted of 15 mice that received pure water. A total of 3 days after initiation of administration, the left maxillary second molar was ligated with a 5-0 silk thread for 7 days. Blood biochemical tests were performed to monitor the systemic effects of AGE. Alveolar bone loss was measured morphometrically using a stereomicroscope, and reverse transcription-quantitative PCR was performed to assay mRNAs of proinflammatory cytokines in gingival tissues. A histological survey was also performed to identify osteoclasts in periodontitis lesions (five mice per group). The total protein and albumin levels showed no significant differences between the AGE and control groups. However, ligation-induced bone resorption was lower in the AGE group than in the control group (P=0.01). Additionally, ligature increased the mRNA expression of inflammatory cytokines, whereas AGE administration tended to suppress them. Remarkably, tumor necrosis factor gene expression was significantly suppressed (P=0.04). The number of osteoclasts in periodontitis lesions was reduced in the AGE-treated group. These results indicate that AGE prevents alveolar bone loss by suppressing the inflammatory responses related to osteoclast differentiation in the periodontal tissue. Further research is needed to elucidate the role of AGE in reducing inflammatory bone resorption.

Introduction

Periodontitis is one of the most common diseases worldwide (1,2) and is caused by chronic bacterial infection around the teeth (3). Disease progression is characterized by chronic inflammatory tissue destruction, especially resorption of the alveolar bone, which supports the teeth (4). Furthermore, this disease is associated with numerous systemic diseases, such as diabetes mellitus and non-alcoholic steatohepatitis, because of periodontitis-related bacterial infections and inflammatory responses (5). Thus, numerous attempts have been made to reduce bacterial infections and chronic inflammation (6). However, these attempts must be made over a long period, possibly over the entire life stage of the individual. Phytochemical reagents, including functional foods and/or dietary supplements, can be suitable for this purpose since they have mild effects and can be consumed daily.

In our previous studies, some dietary phytochemicals that can reduce oral biofilm formation (7,8) and specialized pro-resolving mediators derived from omega-3 fatty acids, such as resolvin D2, that can regulate chronic inflammation to induce tissue regenerative phases, were investigated (9). In addition to these direct reactions, the metabolites of food ingredients have received considerable attention. The health-contributing effects of garlic (*Allium sativum* L.) are well known (10), and a historical review of dietary supplements for lipid-lowering activity has also highlighted the effects of

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Abbreviations: AGE, aged garlic extracts; IL, interleukin; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; NF-κB, nuclear factor kappa-B; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; S1PC, S-1-propenylcysteine; SAC, S-allyl-cysteine; SAMC, S-allyl-mercapto-cysteine; TNF, tumor necrosis factor; TRAP, tartrate-resistant acid phosphatase

Key words: AGE, experimental periodontitis, bone resorption, inflammation, osteoclasts

garlic (11). Even snacks containing garlic extracts have been discussed for health promotion (12), thereby guiding the industrial utilization of garlic in food products and indicating its suitability for the development of functional foods and/or dietary supplements (13). Numerous studies have supported the health benefits of garlic-related phytochemical reagents.

Garlic has been shown to improve periodontal disease in humans (14,15) and dogs (16). Zini *et al* (14,15) conducted two clinical intervention studies and showed that oral intake of aged garlic extract (AGE) prepared using a water-ethanol extraction method prevented periodontitis. Furthermore, Takahashi *et al* (16) showed that AGE supplementation improved gingivitis and halitosis in Beagle dogs. The mechanisms underlying the action of AGE include antioxidant activity (17,18), improvement of peripheral circulation (19), reduction of inflammation (20,21) and enhancement of immune reactions (22,23). Moreover, previous studies have reported that AGE reduces *Porphyromonas gingivalis*-derived lipopolysaccharide (LPS)-induced cellular responses in gingival epithelium cells (24) and fibroblasts (25). These mechanisms of AGE action are considered to be caused by the sulfur components of AGEs, such as S-allylcysteine (SAC), S-1-propenylcysteine (S1PC) and S-allyl-mercapto-cysteine (SAMC). AGE can also be expected to have certain benefits in preventing periodontitis; however, caution should be exercised when utilizing these purified sulfur components. Despite studies highlighting the effective utilization of AGEs containing these compounds in various amounts, the mechanism underlying the preventive and therapeutic effects of AGE-containing functional foods and/or dietary supplements for periodontal disease requires elucidation (26).

The present study aimed to investigate how AGE modifies periodontal conditions, such as alveolar bone resorption, inflammatory reactions and histological changes, in a mouse model of ligature-induced experimental periodontitis.

Materials and methods

Preparation of AGE. AGE was produced by soaking sliced garlic cloves in ethanol/water for >10 months at room temperature (27).

Animal and experimental periodontitis. The animal experiment was conducted using 40 male mice (C57BL/6J: CLEA Japan, Inc.; average weight: 21.5 g at 7 weeks of age). The housing conditions were constant temperature at 23±1˚C with a 12/12-h light/dark cycle, and the mice were provided free access to sterile food (Rodents Diets MF, solid, 12 mmϕ pellet, Oriental Yeast) and water under specific pathogen-free conditions (28). Each mouse received AGE at a safe and sufficiently effective dose of 2 g/kg (28-30) orally using a feeding needle (ball diameter 2.0 mm; As One; https://www.as-1.co.jp/en/) under handholding without anesthesia, starting 3 days before induction of experimental periodontitis and ending 6 days after induction. Pure water was used as the negative control. Animal care and experiments were performed in accordance with Okayama University's Guidelines for the Care and Use of Laboratory Animals, which were reviewed and approved (approval no. OKU-2022934) by the Animal Care and Use Committee of Okayama University (Okayama, Japan).

For induction of experimental periodontitis, the mice were anesthetized with a mixture of 92.48 mg/kg ketamine hydrochloride (Daiichi Sankyo) and 11.184 mg/kg xylazine hydrochloride (Bayer Yakuhin, Ltd.) in phosphate-buffered saline (pH 7.4; Thermo Fisher Scientific, Inc.) by intraperitoneal injection (31). A 5-0 silk ligature was tied around the left maxillary second molar (32). The contralateral tooth was not ligated and served as the baseline control. A total of 7 days after ligation, mice were euthanized using carbon dioxide gas (volume displacement rate of CO_2 was 30%/min).

Observation of alveolar bone resorption. A dissecting microscope (1.2; SZ61; Olympus Corporation) was used to morphologically evaluate the relationship between the bone and tooth root in the debrided maxilla. After bleaching with 31% hydrogen peroxide (Santoku Chemical Industries, Co., Ltd.) followed by staining with 0.5% eosin (Merck KGaA) and 1% methylene blue (Muto Pure Chemicals, Co., Ltd.), the distance from the cementoenamel junction to the alveolar bone crest was measured at three predetermined points on the ligated second molar and two predetermined points on the affected first molar (32). The third molar was excluded from the measurements because they had dropped out in some mice.

Reverse transcription-quantitative PCR (RT-qPCR). Gingival tissues (1x2 mm) were obtained from the palatal side of both molars. Total RNA was extracted using a RNeasy Plus Mini kit (Qiagen GmbH) and quantified using a microvolume spectrophotometer at 260 and 280 nm (NanoDrop-2000; Thermo Fisher Scientific, Inc.). The RNA was reverse transcribed to cRNA by using SuperScript™ IV VILO™ Master Mix (Thermo Fisher Scientific, Inc.) at 25˚C for 10 min, 42˚C for 60 min, and 85˚C for 5 min. qPCR with the cDNA was performed using Power SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Inc.) and specific primers (Table SI) using 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. PCR reaction mixture was kept at 50˚C for 2 min, then at 95˚C for 10 min. PCR cycles were 50 cycles of denaturing at 95 ˚C for 15 sec and annealing and extension at 60˚C for 60 sec. Data were analyzed using the comparative $2^{-\Delta\Delta Cq}$ method (33). GAPDH was used as a reference gene.

Histological evaluation. Mouse maxillae and the surrounding periodontal tissue were fixed in 4% paraformaldehyde phosphate buffer solution (Nacalai Tesque, Inc.) for 24 h at 4˚C, decalcified in 10% ethylene-diamine-tetra-acetic acid solution (pH 7.0, Muto Pure Chemicals Co., Ltd.) for 1 week at 4˚C, and then embedded in paraffin. Tissue samples were sectioned at 7-μm thickness in the coronal direction along the long axis of the teeth. Sections were stained with hematoxylin (Mayer's hemalum solution, Merck KGaA) and eosin (0.5% aqueous eosin Y-solution, Merck KGaA). Osteoclasts were visualized using a tartrate-resistant acid phosphatase (TRAP) staining kit (FUJIFILM Wako Pure Chemical Corporation). Stained images were observed and captured using a microscope under natural light (fluorescence microscope BZ-X800, Keyence Corporation; 1,920x1,440 pixels). TRAP-positive cells with large or multiple nuclei were automatically counted using a microscope (BZ-X800; Keyence Corporation) in 3-5 sections from each mouse. The average

number of TRAP-positive cells on each mouse's control and ligatured sides was used for statistical analysis.

Statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test for multiple-group comparisons. Two-tailed unpaired Student's t-test was used for two-group comparisons. P<0.05 was considered to indicate a statistically significant difference. All analyses were performed using GraphPad Prism (version 8.4.3; GraphPad Software; Dotmatics).

Results

Effects of AGE feeding on the body conditions of mice. The body weight of each mouse was monitored daily, and almost all mice were stable. Only one mouse in the control group showed a weight reduction of ~5 g 2 days after ligation, but its body weight recovered 5 days after ligation (Fig. S1). Blood tests for total protein and albumin levels were also performed. However, no statistically significant differences were observed between the AGE and control groups (Fig. S2).

Suppression of alveolar bone resorption in AGE-fed mice. The actual alveolar bone level reduced significantly after the 7-day ligation around the second molar in both the AGE and control groups (Fig. 1A and B). This reduction was lower in the AGE group than in the control group (P<0.001 in the AGE group, P<0.0001 in the control group; Fig. 1B). However, the difference in this reduction between the two groups was not significant (P=0.14).

Using the difference in actual measurements between the ligated and un-ligated sites in individual mice, ligature-induced bone resorption was compared between the AGE group and the control group. The reduction of bone level in the AGE group was lower than that in the control group (P<0.05; Fig. 1C).

Suppression of mRNA expression of inflammatory cytokines in AGE-fed mice. The mRNA expression of inflammatory cytokines in the periodontitis tissue of the control and AGE groups was compared between the un-ligated and ligated sites (Fig. 2). The expression of these mRNAs at the ligated sites was greater than that at un-ligated sites, especially in the control group. Although these mRNAs were also more highly expressed in the ligated sites than in the un-ligated sites in the AGE group (P<0.05; Fig. 2), the expression of the tumor necrosis factor gene (*TNF*) in the AGE group was lower than that in the control group (P<0.05; Fig. 2).

Reduced number of osteoclasts in ligature-induced periodontitis tissue in AGE-fed mice. Around the periodontal tissue surrounding the ligature, the interdental papillae and alveolar bone crests were destroyed, and cells had infiltrated these areas (Fig. 3A). These phenomena were observed in both the control and AGE groups (Fig. 3B). TRAP staining revealed more osteoclasts at the ligated sites in the control group than in the AGE group (Fig. 3C and D). The number of TRAP-positive osteoclasts increased on the ligature side in both control and AGE-fed mice. However, under ligature-induced periodontitis conditions, it decreased when the mice were fed AGE (Fig. 3E). Even under un-ligated conditions, it also decreased with AGE feeding.

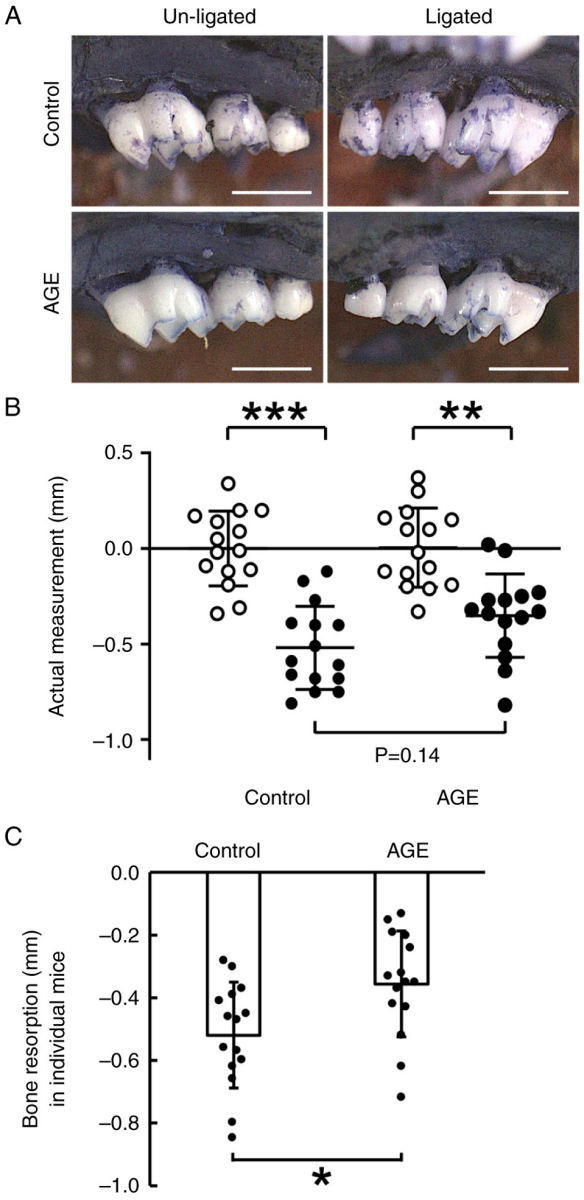

Figure 1. Effects of AGE feeding on alveolar bone resorption in mice with ligature-induced experimental periodontitis mice. (A) Typical palatal views of maxillae stained with 0.5% eosin and 1% methylene blue. The large-sized teeth are the first molars, medium-sized teeth are the second molars, and small-sized teeth are the third molars. Scale bar, 1 mm. (B) Comparison of the actual measurement of the distance from the cementoenamel junction to the alveolar bone crest between the control group and the AGE groups. The left maxillary second molar was ligated, and the contralateral tooth (right) was not ligated in the same mouse in each group (control and AGE groups; n=15 in each group). The baseline (0 mm) was set from the measurement average in un-ligated sites of the control group. Open circle: un-ligated site, closed circle: ligated site. (C) Comparison of the bone resorption calculated in individual mice between the control group and the AGE groups. Bone resorption was calculated as the difference in actual measurement between the un-ligated and ligated sites in individual mice. Data are presented as the mean ± SD of 15 samples for each condition. One-way ANOVA, Tukey's multiple-comparison test (B) and Student's t-test (C) were performed. *P<0.05, **P<0.01 and ***P<0.001. AGE, aged garlic extract.

Discussion

AGE has been reported to show suppressive effects in inflammatory periodontal diseases (14-16). Therefore, in the present study, its effects were evaluated on alveolar bone resorption.

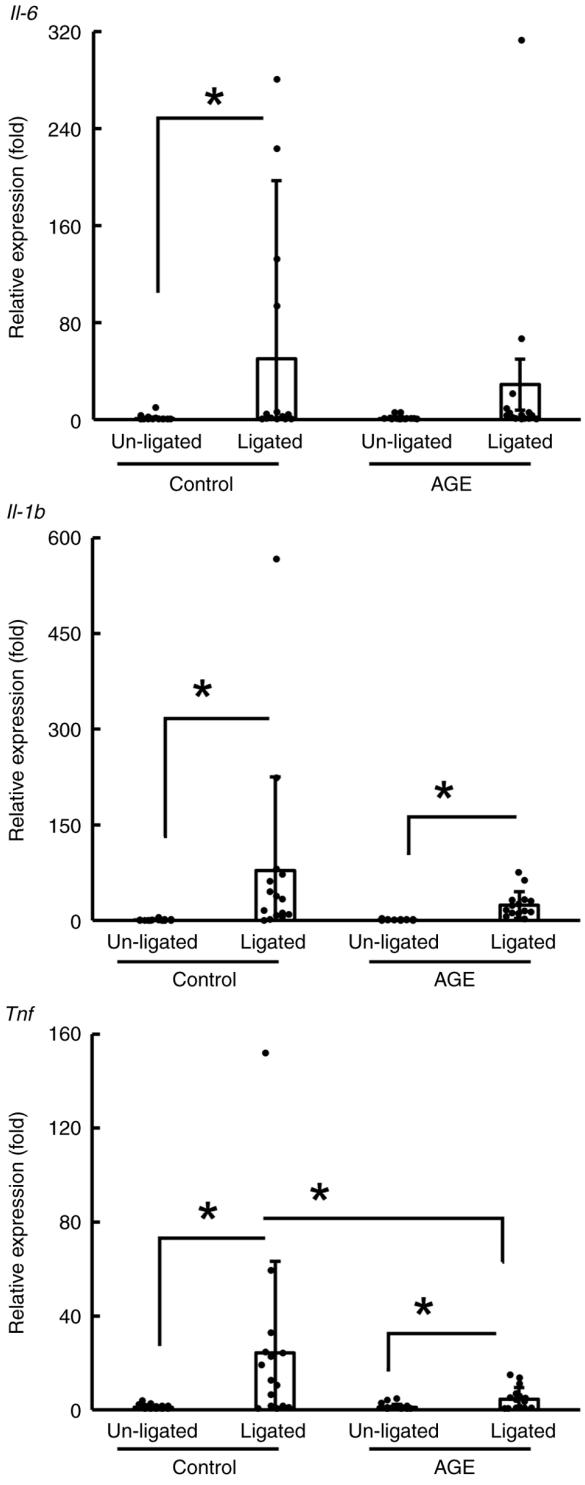

Figure 2. mRNA expression of pro-inflammatory cytokines in mouse palatal gingiva. Reverse transcription-quantitative PCR was performed to analyze the mRNA expression levels of IL-6, IL-1b and TNF. Data are expressed as the mean ± SD of the 15 samples for each condition. mRNA expression levels were normalized to those of GAPDH. One-way ANOVA and Tukey's multiple-comparison tests were performed. *P<0.05. AGE, aged garlic extract.

It was demonstrated that AGE inhibited alveolar bone resorption in an experimental periodontitis model (Fig. 1). In the inflamed periodontal tissue, the expression of inflammatory cytokine genes (especially TNF) was suppressed (Fig. 2). Furthermore, the number of osteoclasts in the inflamed periodontal tissue was reduced when AGE was fed to experimental mice (Fig. 3). These results suggest that AGE suppresses inflammation and osteoclast differentiation, leading to reduced alveolar bone resorption.

The anti-inflammatory effects of garlic have been reported previously (12). In particular, AGE has been shown to reduce interleukin (IL)-6 production in both clinical trials (34,35) and *in vitro* cell culture models (24). Since IL-6 is known to be involved in periodontitis (36,37), the regulation of IL-6 production by AGE is a notable finding. Furthermore, AGE as well as garlic extracts and derivatives may suppress numerous inflammatory processes. Thiosulfinate-enriched garlic extract was shown to inhibit the production of TNF-α, IL-1β and IL-6 in LPS-stimulated monocytes obtained from healthy individuals (38).

Furthermore, allicin, one of the bioactive components of garlic, has been shown to suppress the LPS-induced increases in the levels of inflammatory cytokines, such as IL-1β, IL-6, IL-8 and TNF-α in bovine epithelial cell culture experiments, and to alter nuclear factor kappa-B (NF-κB) signaling pathway (39). In a human gingival cell line, the AGE components SAC and SAMC reduced TNF-α-induced increments in IL-6 production by suppressing NF-κB. In addition, S1PC inhibited the expression of intercellular adhesion molecule-1. AGE may inhibit inflammatory reactions by preventing the migration of inflammatory immune cells and interrupting the mitogen-activated protein kinase signaling pathway (24). In a clinical application of garlic extracts and derivatives for reducing inflammation, a randomized, double-blind clinical trial in patients undergoing peritoneal dialysis demonstrated that administering of 400 mg of garlic extract twice daily for 8 weeks resulted in anti-inflammatory effects on IL-6 and C-reactive protein levels, as well as on the erythrocyte sedimentation rate (40). Unfortunately, no blood test was conducted in the present study. Thus, the effect on blood factors in mice remains unknown. However, thiosulfinate-enriched garlic extract has been suggested to enhance the inflammatory response in monocytes from patients with sepsis, manifesting as increased expression of human leukocyte antigen-DR (38). Thus, the anti-inflammatory effects of garlic extracts and their derivatives may require more careful consideration since they may also have opposite effects. Therefore, garlic extracts and their derivatives should be included in the category of herbal medicines and supplements until their mechanism of action is clarified.

Numerous types of cytokines are involved in osteoclast differentiation (41). Certain cytokines play key roles in the interaction between receptor activator of NF-κB (RANK), its ligand RANKL, and osteoprotegerin, which are crucial for osteoclast differentiation (42). Other cytokines influence the activities of factors such as nuclear factor of activated T-cells and cytoplasmic 1, which promotes osteoclast activation and inhibits osteogenic differentiation factors such as runt-related transcription factor 2 and osterix (43). TNF-α is one of the major cytokines involved in osteoimmunology and stimulates osteoclastogenesis-related other factors such as macrophage colony-stimulating factor (M-CSF) and RANKL, thereby causing pathological bone resorption such as alveolar bone resorption in periodontitis (44). The present results demonstrated that AGE reduced alveolar bone resorption, the

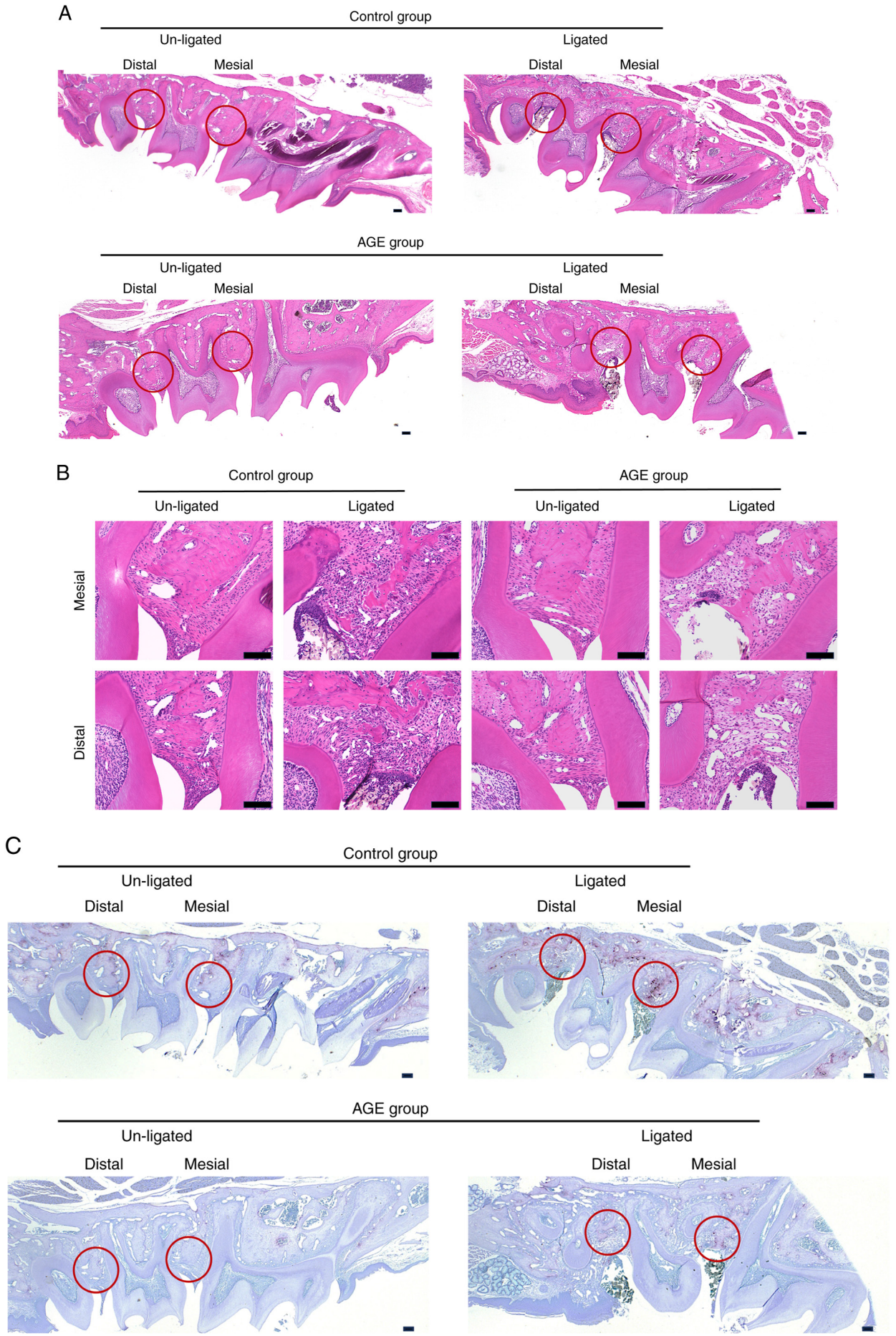

Figure 3. Continued.

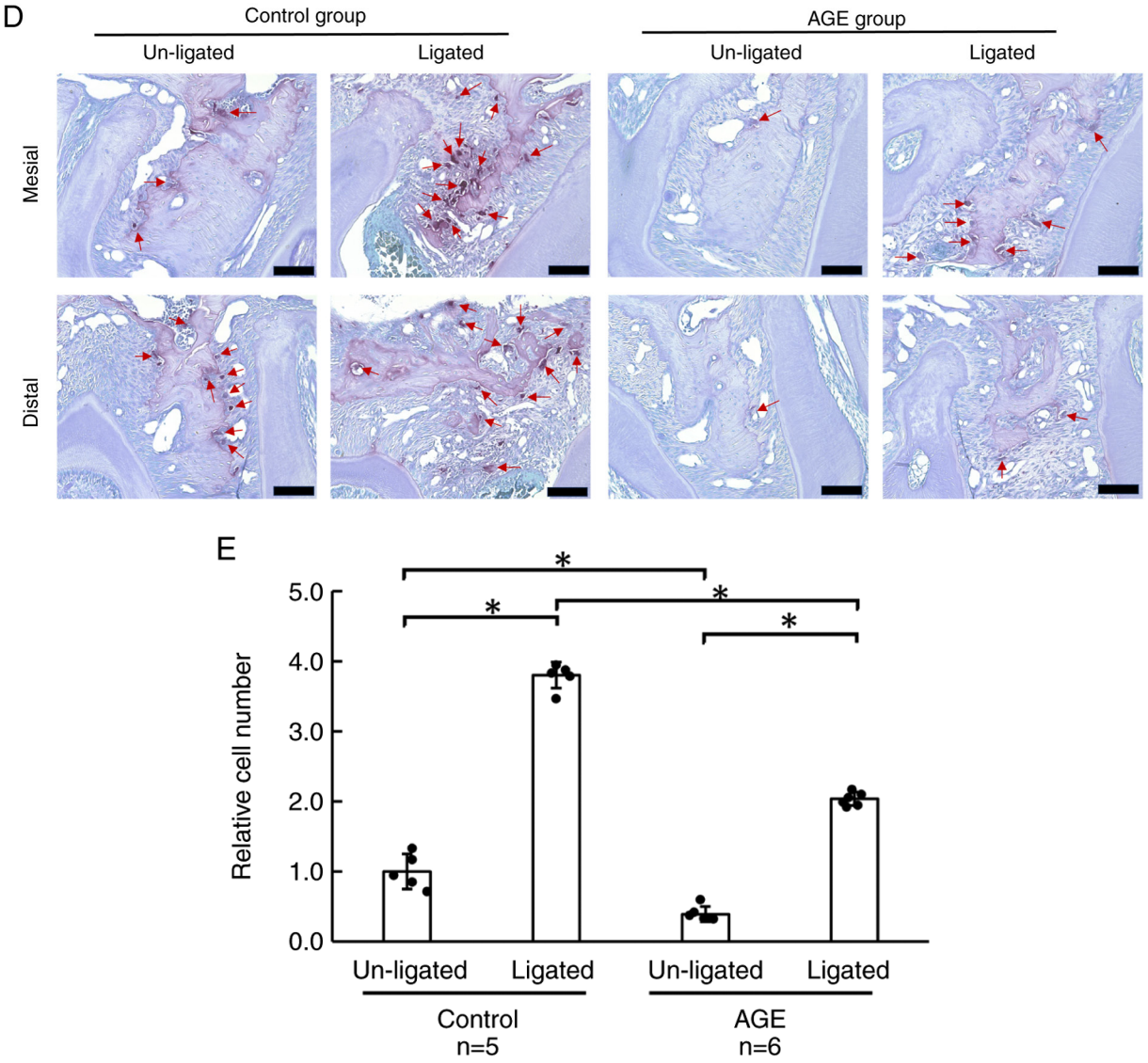

Figure 3. Histological analysis of maxillae and surrounding periodontal tissues in mice with ligature-induced experimental periodontitis. (A and B) Hematoxylin and eosin staining. The enlarged images within the red circles in A are shown in B. (C and D) TRAP staining. The enlarged images within the red circles in C are shown in D. Representative sections for each condition (control group: n=5, AGE group: n=6) are shown using serial sections. Red arrow: osteoclasts. Scale bar, 100 μm. (E) The mean and standard deviations of the relative numbers of TRAP-positive cells on the un-ligated and ligated sides of each mouse. After analysis using one-way ANOVA followed by Tukey's multiple-comparison test, a two-tailed Student's t-test was used for two-group comparisons. *P<0.05. TRAP, tartrate-resistant acid phosphatase; AGE, aged garlic extract.

expression level of TNF mRNA, and the number of osteoclasts in periodontal tissue, suggesting that further investigation is needed to clarify osteoclastogenesis, especially TNF-α secretion from monocytes/macrophages, and effects on the expression of factors related to osteoclastogenesis.

There are certain limitations to the present study. The suppressive effect of AGE on alveolar bone resorption was not as strong as the average bone level, which was evident in AGE-fed mice (Fig. 1B; P=0.14 between control and AGE groups at ligature sites). These effects were observed when bone levels were compared in the individual mice (Fig. 1C). This suggests that AGE does not exert any apparent suppressive effects on bone resorption, like certain bisphosphonate derivatives. This weak suppressive effect may be related to the signaling pathways that induce osteoclast differentiation. However, the present study evaluated only three inflammatory cytokines. Further studies are needed to assess inflammatory cytokine-related osteoclast differentiation signaling pathways, such as those involving M-CSF and RANKL. Further experiments on how AGE affects osteoclast differentiation are conducted by the authors based on the results of the present study. The involvement of reactive oxygen species is another topic of further research (45). Aged black garlic extract has been reported to have higher antioxidant activity than fresh raw garlic extract (46). This represents another perspective worth exploring in future studies. A series of studies on AGE-induced improvement of osteoimmunology is needed to expand the potential applications of AGE in healthy living (12).

In conclusion, AGE prevented alveolar bone loss by suppressing the expression of inflammatory cytokines related to osteoclast differentiation in periodontal tissues. Further studies are needed to elucidate how AGE alters the secretion of osteoclastogenesis-related cytokines in inflamed periodontal tissues.

Acknowledgements

The authors would like to thank Dr Toshiaki Matsutomo (Wakunaga Pharmaceutical Co., Ltd.) for his helpful advice, encouragement, and critical evaluation of this manuscript. The authors would also like to thank Drs Jun Sakurai and Yasuhiro Fujii (Center for Innovative Clinical Medicine, Okayama University Hospital, Japan) for organizing and supporting the collaborative research.

Funding

The present study was supported by the Collaborative Research Contract (grant no. 7262000084) between Okayama University and Wakunaga Pharmaceutical Co., Ltd.

Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CK conceptualized the study, developed methodology, performed formal analysis and investigation, and wrote the original draft. AH conceptualized the study, developed methodology, validated data, performed investigation and visualization, provided resources and supervised the study. CKN developed methodology and conducted investigation. HN and MO performed formal analysis, data curation and validation. KO validated data, supervised the study, wrote, reviewed and edited the manuscript. ST conceptualized the study, validated data, reviewed and edited the manuscript, performed project administration and acquired funding. CK, AH and ST confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal care and experiments were performed in accordance with Okayama University's Guidelines for the Care and Use of Laboratory Animals, which were reviewed and approved (approval no. OKU-2022934) by the Animal Care and Use Committee of Okayama University (Okayama, Japan).

Patient consent for publication

Not applicable.

Competing interests

MO and HN are employed by Wakunaga Pharmaceutical Co., Ltd., who provided the funding for the present study. Specifically, the funder provided the AGE for the present study.

Use of artificial intelligence tools

During the preparation of this work, artificial intelligence tools were used to improve the readability and language of the manuscript or to generate images, and subsequently, the authors revised and edited the content produced by the artificial intelligence tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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