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Effects of Coffee Intake on Oxidative Stress During Aging-related Alterations in Periodontal Tissue

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Abstract. *Background/Aim: The purpose of this study was to determine the anti-aging effects of coffee intake on oxidative stress in rat periodontal tissue and alveolar bone loss. Materials and Methods: Male Fischer 344 rats (8 weeks old) were randomized to four groups; the baseline group immediately sacrificed, the control group fed with normal powdered food for 8 weeks, and the experimental groups fed with powdered food containing 0.62% or 1.36% coffee components for 8 weeks. Results: Alveolar bone loss and gingival level of 8-hydroxydeoxyguanosine were significantly lower in the 1.36% coffee group than in the control group. Nuclear factor erythroid 2-related factor 2 translocation to the nucleus was significantly higher in the 1.36% coffee group than in the control group. Conclusion: Continuous intake of 1.36% coffee could prevent age-related oxidative stress in the periodontal tissue and alveolar bone loss, possibly by up-regulating the Nrf2 signaling pathway.*

Aging is defined in biological terms as an age-dependent decline in physiological function (1) and characterized by an increased risk of age-associated diseases and death. One potential cause of aging is mitochondrial dysfunction and oxidative damage (2, 3). Biosynthesis, oxidative phosphorylation ability, and other activities of mitochondria decline with aging, and mitochondrial DNA damage, apoptosis induction and production of reactive oxygen species (ROS) progressively increase (4, 5). Dysfunction of the mitochondrial electron transport system leads to increased ROS production, which is considered as an important factor in accelerating the aging process (6). Therefore, reducing ROS *via* antioxidant agents might help suppress the aging process.

Aging is closely associated with alveolar bone loss (ABL); odds ratios (OR) range from 1.72 for 35-44-year-old persons to 9.01 for 65-74-year-olds (7). The fact that ROS play a role in periodontal tissue destruction has also been identified (8-10). On the other hand, animal and clinical studies have found that antioxidant agents can help prevent periodontal disease (11, 12). These studies focused on the effects of short-term antioxidant agent intake on periodontal disease. However, the effects of continuous intake of antioxidant agents on aging over the longer term remain unclear.

Coffee is a popular beverage consumed in many countries and its ingredients have a powerful antioxidant capacity (13). An animal study showed that coffee improved liver oxidative balance in rats fed high fat diet (14). Also, an epidemiological study has found an inverse association between coffee consumption and severe periodontitis (15). On the other hand, some studies showed that coffee and some of its components might have detrimental effects on periodontal tissues (16-18). The mechanism through which coffee components affect the aging of periodontal tissue is unknown.

Herein, we postulated that coffee intake would suppress age-related oxidative stress in periodontal tissue and ABL. Therefore, the purpose of this study was to evaluate the effects of coffee intake on oxidative stress in rat periodontal tissue and ABL.

Materials and Methods

Animals. Male Fischer 344 rats (8 weeks old, n=32) were used in this study. The rats were housed in an air-conditioned room (23-25˚C) with a 12 h light-dark cycle. The experiments were performed in accordance with the institutional guidelines of the Animal Care

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and Use Committee, Okayama University (OKU-2016248) according to the ARRIVE and 3Rs statements.

Experimental design. The rats were randomly assigned to four groups (n=8 each). The baseline group was immediately sacrificed and the control group was fed with MF normal powdered food (Oriental Yeast Co. Ltd., Osaka, Japan) for 12 weeks. The experimental groups (0.62% and 1.36% coffee groups) were fed with MF normal powdered food containing 0.62% or 1.36% freeze-dried, powdered coffee components (Nescafé Excella L906320:16; Nestlé Japan Ltd., Kobe, Japan) for 12 weeks, respectively (19). This coffee component contains 150mg/g polyphenol, and the proportion of chlorogenic acid in the polyphenol is 15%.

The rats were euthanized under isoflurane inhalation at the conclusion of the 12-week experimental period. The right maxillary molar regions were resected *en bloc*, fixed in Bouin's fluid for one day and decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) at room temperature for two weeks for histological analysis. Gingival biopsy samples of the left maxillary molar regions were homogenized using a frozen cell crusher (Microtec Co., Chiba, Japan) for real-time polymerase chain-reaction (PCR) investigations. The right mandibular regions were resected to measure alveolar bone loss. Serum was separated by centrifugation from cardiac blood at 1,500 × *g* for 15 min and stored at –80˚C.

Measurements of serum oxidative stress and antioxidant capacity. Serum levels of reactive oxygen metabolites (ROM) and total antioxidant capacity were determined by spectrophotometry using a Free Radical Elective Evaluator (Diacron International, Grosseto, Italy) as described (9, 20). Serum levels of ROM are markers of circulating ROS, which are expressed in Carratelli units (CARR U). One CARR U corresponds to 0.08 mg/dl hydrogen peroxide. The OXY-adsorbent test evaluates the capacity of serum to counteract the oxidative action of hypochlorous acid (HClO), and results are expressed as micromoles of HClO consumed by 1 ml of test sample (μmol HClO/ml).

Histological and immunohistochemical analyses. Bucco-palatal sections (2 μm thick) were dehydrated with ethanol, immersed in xylene and embedded in paraffin, then stained with hematoxylin and eosin (HE) or immunostained for 8-hydroxydeoxyguanosine (8-OHdG) using a commercial kit (Histofine Simple Stain MAX PO; Nichirei Co., Tokyo, Japan). The hydroxyl product of deoxyguanosine produced by oxidative stress, 8-OHdG, is generally regarded as a reliable indicator of oxidative stress (10, 21). Polyclonal antibodies against 8-OHdG (Chemicon International, Temecula, CA, USA) were diluted 1:200 in phosphate buffered saline. The sections were placed in 3-3'-diaminobenzidine tetrahydrochloride for color development and then counterstained with Mayer's hematoxylin.

Histological measurements were performed using a×400 microscope. 8-OHdG positive cells and total cells were counted in standard areas (0.1 mm × 0.1 mm each) within the gingiva (the connective tissues subjacent to the junctional epithelium) and periodontal ligament (22). Intra-examiner reproducibility was confirmed by double-scoring 10 randomly selected sections at two-week intervals. Intra-examiner agreement for 8-OHdG-positive cells was >80%.

Measurements of alveolar bone loss. Right mandibular regions were de-fleshed using curettes, stained with 1% aqueous methylene blue (Sigma, St. Louis, MO, USA) for five minutes and photographed using a digital camera (Nikon Instruments Inc., Tokyo, Japan). The distance (in mm) from the cement-enamel junction (CEJ) to the alveolar bone crest (ABC) of the first molars (three roots) were determined by image analysis using ImageJ software (NIH, Bethesda, MD, USA). The average ABL at three points was defined as reflecting the amount of alveolar bone loss (23).

Gene expression analysis using real-time reverse transcription-polymerase chain reaction array (RT-PCR array). Total RNA was extracted from pooled gingival samples (n=3 per group) using the TRI reagent (Molecular Research Center Inc., Montgomery, OH, USA). The purity of the RNA samples (A260/A230 ratio, >1.8; A260/A280 ratio, 1.8-2.0) and concentrations (>100 ng/μl) were confirmed and then the RNA was reverse transcribed. First-strand complementary DNA was prepared from total RNA (0.8 μg) using the RT2 First-Strand Kit (Catalogue no., 330401; Qiagen, Hilden, Germany). The reverse transcription reaction proceeded at 37˚C. In brief, 0.8 μg of total RNA was added to 2 μl of Buffer GE (5Å~ gDNA Elimination Buffer), and the final volume was adjusted to 10 μl with RNase-free water. The mixture was denatured at 42˚C for 5 min, cooled on ice for 60 seconds and reverse transcribed after adding 10 μl of reverse transcription mix to the solution. The reaction mixture was incubated at 42˚C for 15 min, and then terminated by heating at 95˚C for 5 min. The cDNA samples generated (20 μl) were then diluted with 91 μl of RNase-free water and stored at –20˚C. Real-time PCR proceeded using a Rotor-Gene 6000 Real-Time PCR detection system (Qiagen). Gene expression was examined using the Rat Oxidative Stress and Antioxidant Defense RT2 Profiler™ PCR Array (Qiagen). The expression of 84 different genes was targeted for detection by real-time PCR. The RT2 Profiler™ PCR Array includes integral undercoating primers for 84 tested and 5 housekeeping genes as well as positive control elements to determine the efficiency of the reverse transcription reaction, the capacity of the PCR reaction, and detection of genomic DNA contamination. The PCR mixture for 100 reactions contained 1,150 μl of SYBR Green ROX FAST Mastermix (Qiagen), 102 μL of cDNA template, and 1,048 μl of RNase-free water. The PCR reaction mix was added to the wells of the PCR plate in equal amounts (20 μl), then the real-time PCR cycling program of 10 min at 95˚C and 40 cycles of denaturation at 95˚C for 15 s recommended by the plate manufacturer for Rotor-Gene 6000 was followed by annealing for 30 s and elongation at 60˚C. Melting curves were subsequently analyzed using Rotor-Gene Q (version 2.1.0; Qiagen) software. Fold changes in each gene in the 1.36% coffee group were compared with gene expression by the control group.

Histological evaluation of nuclear factor erythroid 2-related factor 2 (Nrf2) translocation. We double-stained tissue sections with fluorescence to confirm Nrf2 translocation to the nucleus. Antigen was retrieved using Histo VT One (Nacalai Tesque, Kyoto, Japan) at 98˚C for 40 min followed by incubation for 20 min at room temperature. A polyclonal antibody against Nrf2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) was diluted to 1:500 in phosphate-buffered saline (3). The secondary antibody was 1:250-diluted Alexa Fluor 594-conjugated anti-rabbit IgG (Thermo Fisher Scientific, Kanagawa, Japan), which produces red fluorescence with excitation and emission maxima of 561 and about 594 nm respectively (24). Thereafter, the slides were covered with 4', 6-diamidino-2-phenylindole (DAPI) mounting medium (ImmunoSelect Antifading Mounting Medium; Dianova, Hamburg, Germany), which produces blue fluorescence with excitation and emission maxima of 365 and about 460 nm, respectively (25).

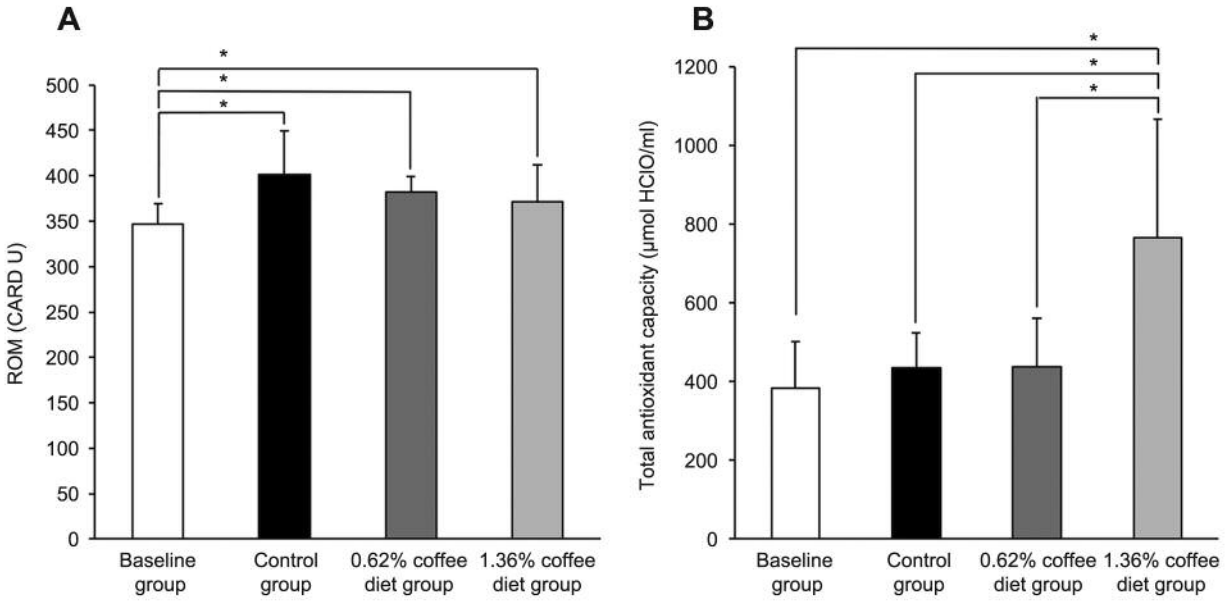

Figure 1. *Serum levels of ROM (A) and total antioxidant capacity (B). Bars represent mean±SD (*p<0.05, according to one-way ANOVA followed by Tukey's method).*

Statistical analysis. The data are presented as the mean values±standard deviation. The significance of differences between the groups was evaluated by one-way ANOVA followed by Tukey's method or Student's *t*-test using a statistical software package (SPSS version 22.0; IBM, Tokyo, Japan). The level of significance was set at $p<0.05$. Intra-examiner agreement was more than 80% in all experimental procedures.

Results

Food consumption did not significantly differ between the control and coffee groups during the experimental period (data not shown). Also, the average body weight did not significantly differ among three groups at baseline and after 12 weeks (control group; 174.8 g and 307.0 g, 0.62% coffee group; 177.2 g and 315.5 g, 1.36% coffee group; 171.6 g and 311.1 g, respectively).

Serum levels of ROM were significantly lower in the baseline group than in all other groups ($p=0.023$). Serum levels of ROM were lower in the 0.62% and 1.36% coffee groups than in the control groups, but the differences did not reach significance (Figure 1A). On the other hand, serum levels of total antioxidant capacity were significantly higher in the 1.36% coffee group than in the baseline, control and 0.62% coffee groups ($p=0.001$, 0.005 and 0.005, respectively; Figure 1B).

The ratios of 8-OHdG-positive cells to total cells were significantly higher in periodontal tissues from the control, than the baseline group ($p<0.001$). The ratios of 8-OHdG-positive cells to total cells in the gingival tissue in the 1.36% coffee group were significantly lower than those in the control and 0.62% coffee groups ($p=0.001$ and 0.018, respectively; Figure 2).

The mean gap between the CEJ and the ABC was significantly greater in the control than in the baseline group ($p<0.001$), and that between CEJ and the ABC was significantly lower in the 1.36% coffee group than in the control group ($p=0.001$; Figure 3).

RT-PCR analysis identified three genes with over a two-fold increase in expression in the 1.36% coffee group compared with the control group. Glutamate cysteine ligase modifier subunit, ferritin, and hypoxanthine phosphoribosyltransferase 1 mRNA values were elevated (Table I).

Nuclear levels of Nrf2 were significantly higher in the 1.36% coffee group than in the control group according to nuclear translocation assays ($p=0.008$; Figure 4).

Discussion

To the best of our knowledge, this is the first study to investigate the effects of continuous coffee intake on gingival oxidative stress and ABL in rats. The ratios of 8-OHdG-positive to total cells in periodontal tissues were significantly higher in the control group at 20 weeks of age than in the baseline group at 8 weeks of age. Since 8-OHdG is a parameter of oxidative DNA damage (10, 21), these findings indicated that oxidative stress in periodontal tissue increases

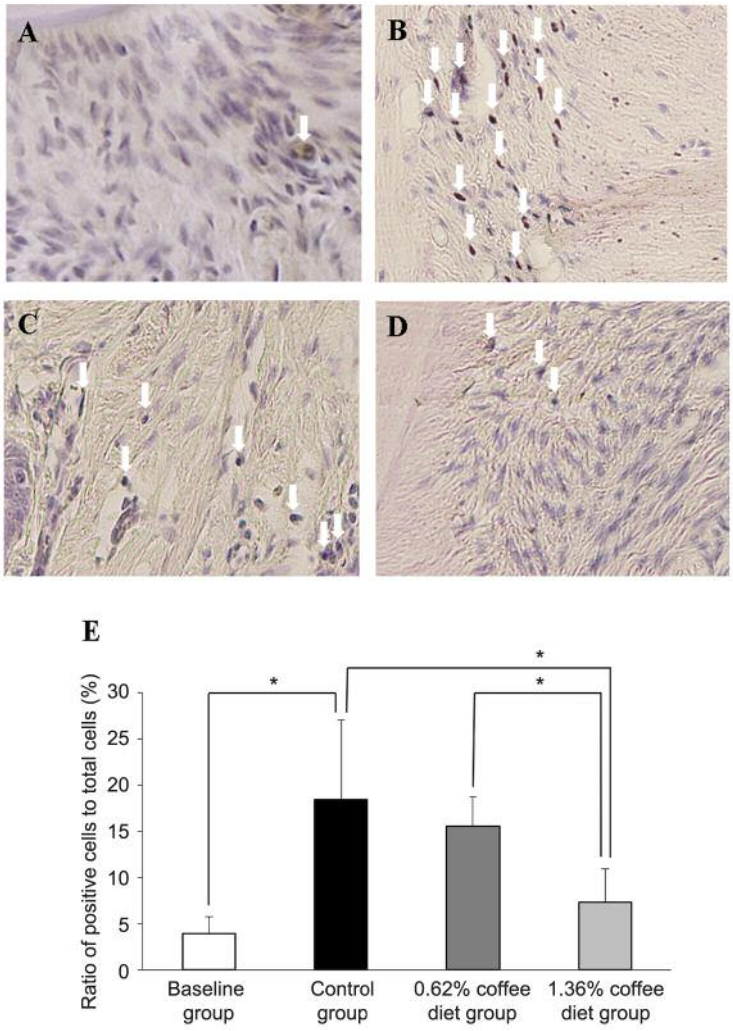

Figure 2. *8-OHdG-positive cells (white arrows) in the gingival tissue (A: Baseline group, B: Control group, C: 0.62% coffee diet group, D: 1.36% coffee diet group). Ratios of 8-OHdG-positive cells to total cells (E). Bars represent mean±SD (*p<0.05, according to one-way ANOVA followed by Tukey's method).*

with aging. In contrast, the ratios of 8-OHdG-positive to total cells in the periodontal tissue were significantly lower in the 1.36% coffee group than in the control group. Serum values for total antioxidant capacity were significantly higher in the 1.36% coffee group than in the control group. Continuous intake of a diet containing 1.36% coffee might reduce age-related oxidative stress in periodontal tissues by increasing systemic antioxidant capacity.

Serum levels of ROM were significantly higher in the control, than in the baseline group, indicating that systemic oxidative stress increases with aging. In contrast, serum levels of ROM were concentration-dependently lower in the 0.62% and 1.36% coffee groups than in the control group, but the differences did not reach statistical significance. A previous study identified a significant reduction in serum ROM in rats after continuous intake of hydrogen-rich water for >10 months (26). Our model involved continuous coffee intake for 12 weeks, which might have been insufficient to reduce systemic oxidative stress.

The distance from the CEJ to the ABC was significantly greater in the control group at 20 weeks of age compared to the baseline group at 8 weeks of age, and significantly lower in the 1.36% coffee group than in the control group. Oxidative stress in periodontal tissues is involved in the progression of alveolar bone loss (27, 28). Previous studies have revealed that endothelial nitric oxide synthase, which is synthesizes nitric oxide, regulates osteogenesis by activating osteoblasts (29, 30). Nitric oxide is a free radical with a powerful oxidative effect. We did not determine nitric oxide levels here, but continuous intake of 1.36% coffee

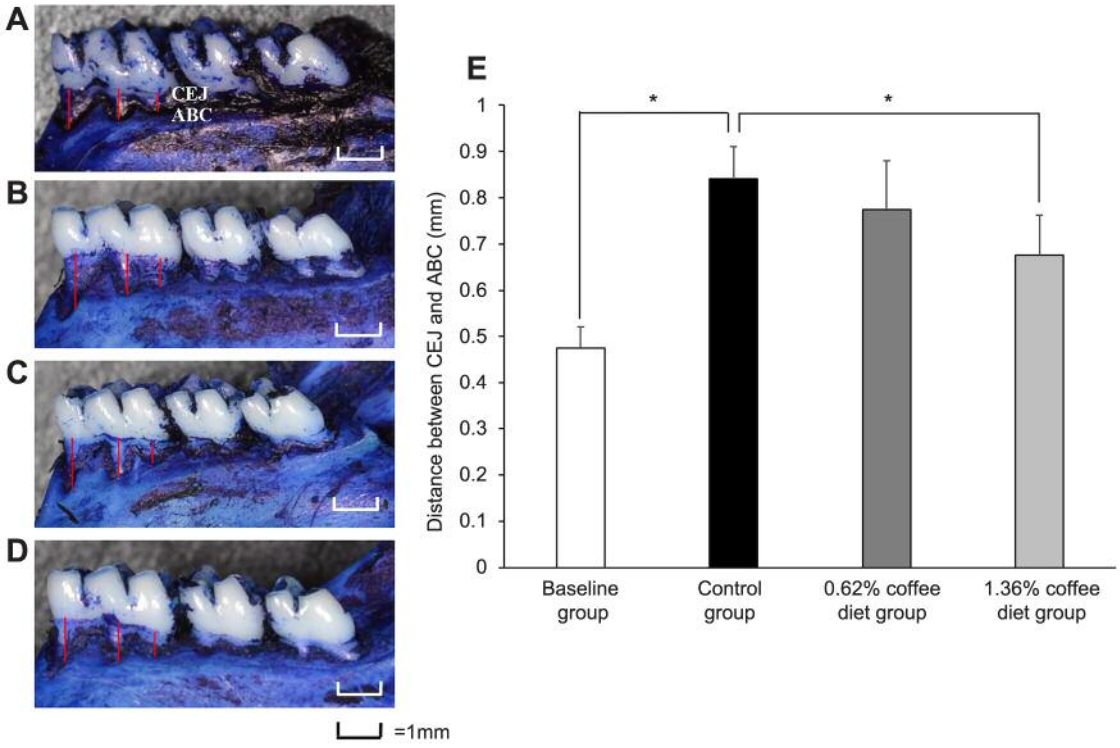

Figure 3. *Alveolar bone loss (red lines) (A: Baseline group, B: Control group, C: 0.62% coffee diet group, D: 1.36% coffee diet group). Distance between the CEJ and the ABC (E) Bars represent mean±SD (*p<0.05, according to one-way ANOVA followed by Tukey's method).*

might suppress alveolar bone loss by inhibiting age-related oxidative stress in periodontal tissues.

High levels of the glutamate cysteine ligase modifier subunit, ferritin, and hypoxanthine phosphoribosyltransferase 1 genes were expressed in the 1.36% coffee group. Glutamate cysteine ligase modifier subunit is a rate-limiting enzyme in the synthesis of glutathione, which is the one of the most abundant intracellular antioxidants (31-33). Ferritin protects cells against iron-dependent oxidative stress and is involved in the regulation of oxidative stress (34, 35). These results suggest that continuous intake of coffee increased the antioxidant capacity of the periodontal tissue, which in turn might contribute to decreasing oxidative stress with aging in these tissues.

The redox-sensitive transcription factor, Nrf2, plays an important role in tissue antioxidant defense (36). Kelch-like ECH-associated protein 1 (Keap1) suppresses Nrf2 in the absence of oxidative stress (37). However, Nrf2 is released from repression mediated by Keap1 when exposed to oxidative stress and transferred into the nucleus to bind antioxidant response elements (38). The present study found more frequent Nrf2 nuclear translocation in the 1.36% coffee group than in the control group (Figure 4). These findings suggest that coffee enhances the antioxidant status of periodontal tissue by upregulating the Nrf2 signaling pathway. This notion is consistent with the previous finding that chlorogenic acid in coffee relieves oxidative stress associated with intestinal inflammation (39).

Table I. *List of the differentially expressed genes between the 1.36% coffee diet group and the control group.*

Genes	Fold change
Glutamate cysteine ligase, modifier subunit	2.38
Ferritin	2.27
Hypoxanthine phosphoribosyltransferase 1	2.23

Coffee contains several antioxidant compounds. Among them, chlorogenic acid is a major coffee polyphenol with a powerful antioxidant capacity (40, 41). Chlorogenic acid significantly decreases malondialdehyde, a product of lipid peroxidation degradation, and significantly increases glutathione, superoxide dismutase and catalase in the livers and kidneys of mouse models of chronic injury (42). Chlorogenic acid inhibits malondialdehyde levels in the hippocampus and frontal cortex of mice (43). It remains

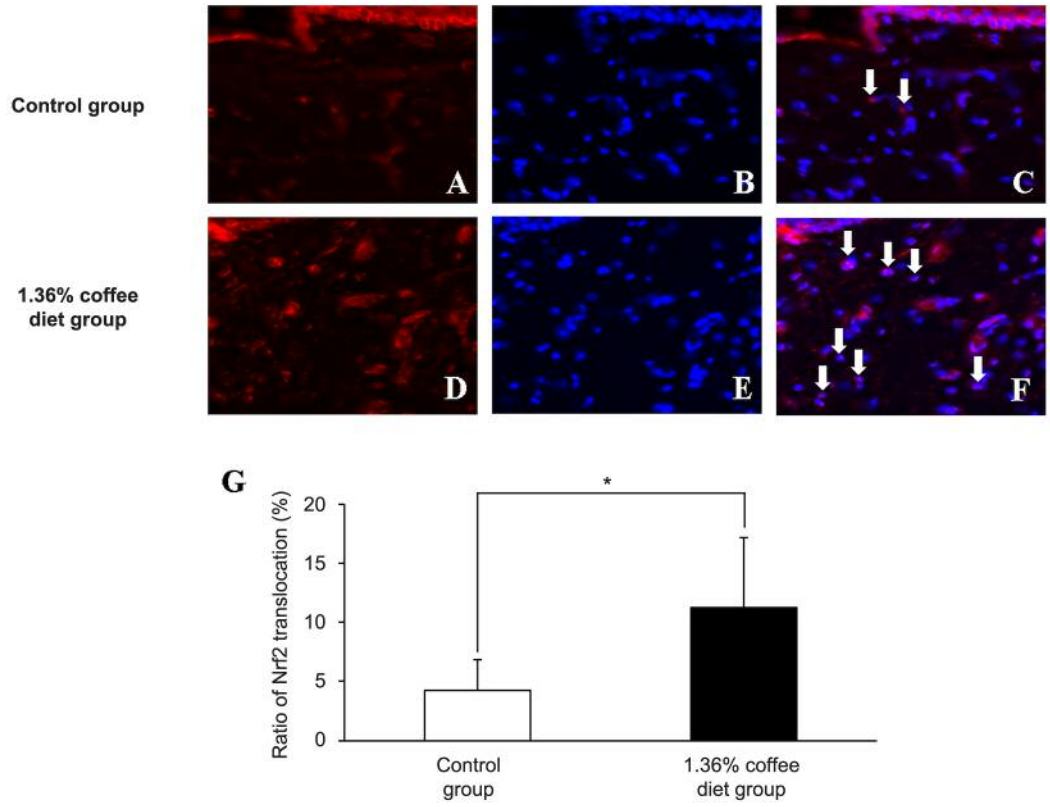

Figure 4. *Translocation of Nrf2 to the nucleus (white arrows) (C, F). The nuclear level of Nrf2 was determined using immunofluorescence staining (red) (A, D). The cell nuclei were visualized by DAPI staining (blue) (B, E). The ratios of Nrf2 translocation (G). Bars represent mean±SD (*p<0.05, according to Student's t-test).*

unclear whether the results of this study were actually due to chlorogenic acid. Nevertheless, the finding that coffee intake decreased periodontal oxidative damage is consistent with these previous results.

This study has certain limitations. Firstly, the experimental period was only 12 weeks. Therefore, the long-term anti-aging effects of coffee intake on oxidative stress in periodontal tissues and alveolar bone loss should be clarified. Also, we started to observe the effects of coffee intake using 8-weeks-old rats. There might be different results when we use older rats. Secondary, this study examined changes in healthy periodontal tissue with increasing age and the effect of continuous coffee intake. The results of the effect of continuous coffee intake on periodontal disease might be different from those of this study. Thirdly, we focused on the anti-aging effect of coffee intake on oxidative stress in periodontal tissues, and did not consider changes in oral microorganisms. A previous study has shown that chlorogenic acid inhibits periodontogenic bacteria such as *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* (44). Changes in periodontogenic microbiota with advancing age should be investigated.

In conclusion, continuous intake of 1.36% coffee prevented age-related oxidative stress in periodontal tissues and alveolar bone loss in rats, possibly by up-regulating the Nrf2 signaling pathway.

Conflicts of Interest

The Authors declare no conflicts of interest.

Authors' Contributions

Terumasa Kobayashi, Takayuki Maruyama, Tetsuji Azuma, Takaaki Tomofuji, Daisuke Ekuni and Manabu Morita conceived and designed the experiments; Terumasa Kobayashi, Hisataka Miyai and Tetsuji Azuma performed the experiments; Terumasa Kobayashi and Takayuki Maruyama analyzed the data; Terumasa Kobayashi, Takayuki Maruyama, Daisuke Ekuni, and Manabu Morita wrote the paper.

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