Anti-aging Effects of Coenzyme Q10 on Periodontal Tissue

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ABSTRACT

Oxidative stress is associated with age-related reactions. The anti-oxidative effects of reduced form of coenzyme Q10 (rCoQ10) suppress oxidative stress, which may contribute to the prevention of age-related inflammatory reactions. We examined the effects of topically applied rCoQ10 on periodontal inflammatory reactions in a rat aging model. Male Fischer 344 rats of 2 months (n= 6) and 4 months (n= 18) of age were used. All of the 2-month-old rats and 6 of the 4-month-old rats were killed immediately. The 12 remaining 4-month-old rats received topically applied ointment with or without 1% rCoQ10 on the gingival surface until they reached 6 months of age. The rats showed an age-dependent increase in circulating oxidative stress. RCoQ10 decreased oxidative DNA damage and tartrate-resistant acid phosphatase–positive osteoclasts in the periodontal tissue at 6 months of age as compared to the control. The same conditions lowered gene expression of caspase-1 and interleukin-1β in the periodontal tissue. Furthermore, Nod-like receptor protein 3 inflammasomes were less activated in periodontal tissues from rCoQ10-treated rats as compared to the control rats. Our results suggest that rCoQ10 suppresses age-related inflammatory reactions and osteoclast differentiation by inhibiting oxidative stress.

INTRODUCTION

Aging is commonly defined as progressive deleterious alterations in various organs and tissues, including inflammatory reactions in periodontal tissue (Chung *et al*., 2011). Although the mechanisms of aging are not understood completely, some of them have been revealed by modern molecular biology. For instance, the autophagic clearance of mitochondria decreases and dysfunctional mitochondria provoke chronic oxidative stress during aging (Salminen *et al*., 2013). Moreover, in the aging process, oxidative stress can stimulate the activation of the Nod-like receptor protein (NLRP)3 inflammasome, which serves as a platform for caspase-1 activation and subsequent proteolytic maturation of the potent pro-inflammatory cytokine interleukin (IL)-1β (Kauppinen *et al*., 2012; Salminen *et al*., 2012). These findings indicate that oxidative stress can stimulate age-related destructive reactions. Therefore, therapeutic approaches against oxidative stress may be effective to suppress age-related destructive reactions.

Coenzyme Q10 (CoQ10) is a vitamin-like, oil-soluble molecule, and its reduced form is a potent lipophilic antioxidant. Studies have demonstrated that CoQ10 has anti-oxidative effects and anti-aging properties at the skin level (Blatt & Littarru, 2011) and in spatial learning (Shetty *et al*., 2012). Therefore, it is possible that CoQ10 also suppresses age-related inflammatory reactions at the periodontal tissue level. Clinical

studies previously showed that CoQ10 improved chronic periodontitis (Hanioka *et al.*, 1994) and salivary secretion (Ryo et al. 2011). However, it is still unclear whether CoQ10 offers clinical benefits to age-related inflammatory reactions in the periodontal tissue.

In the present study, we hypothesized that the anti-oxidative effects of CoQ10 might suppress age-related inflammatory reactions in periodontal tissue. The reduced form of CoQ10 (rCoQ10) has gained attention for its anti-oxidative potential (Littarru & Tiano, 2007). Thus, the purpose of this study was to investigate the preventive effects of topically applied rCoQ10 on age-related inflammatory reactions in rat periodontal tissue. The level of oxidative stress was determined by measuring 8-hydroxydeoxyguanosine (8-OHdG) (Takane et al., 2002; Rai et al., 2011). To gain better insight into the mechanism of action, we analyzed the histological changes, oxidative tissue damage, and NLRP3 inflammasome activation.

MATERIALS & METHODS

Animals

Male Fischer 344 rats that were 2 months (n=6) and 4 months (n=18) of age were housed in an air-conditioned room (23-25°C) with a 12-h light-dark cycle. They had free access to powdered food (MF, Oriental Yeast Co. Ltd., Osaka, Japan) and drinking water. The experimental protocol was approved by the Animal Research Control Committee of Okayama University.

Experimental design

All of the 2-month-old rats (n=6) and 6 of the 4-month-old rats were killed immediately. The remaining twelve 4-month-old rats were randomly divided into two groups of 6 rats each. Under general anesthesia (2%–4% isoflurane delivered in O_2 gas), the experimental group received topically applied ointment containing 1% rCoQ10 (Kaneka Co., Osaka, Japan) on the gingival surface. The control group received topically applied ointment without rCoQ10. The ointment was applied with a cotton ball and did not have any medicinal properties. In both groups, the applied ointment was wiped off after 10 minutes. These treatments were performed 5 times a week until the rats reached 6 months of age.

Sampling

The animals were sacrificed under general anesthesia. For histological analysis, the right maxillary molar regions were resected *en bloc* from each rat and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 1 day. Gingival biopsy samples of the left maxillary molar regions were homogenized by a frozen cell crusher (Microtec Co., Chiba, Japan) and were used to perform real-time polymerase chain reaction (PCR). Blood samples were also collected from the heart. Serum was separated by centrifugation at $1{,}500 \times g$ for 15 minutes and stored at -80°C until analysis.

Histopathological analysis

After fixation with paraformaldehyde, the maxillary samples were decalcified with 10% tetrasodium-EDTA aqueous solution (pH 7.4) for 2 weeks at 4°C. Formalin-fixed tissue samples were embedded in paraffin following dehydration with ethanol (70%, 80%, 90%, and 100%) and immersion in xylene. The paraffin-embedded buccolingual sections of 4-µm thickness from the mesial root area of each first-molar were then stained with hematoxylin and eosin or other stains, as described below. In this study, nine sections from each tooth were randomly selected for staining.

Immunohistochemical staining for 8-OHdG and tartrate-resistant acid phosphatase

(TRAP) staining were performed. Commercial kits (Nichirei Co., Tokyo, Japan) were used to determine the level of 8-OHdG. Polyclonal antibodies against 8-OHdG (Chemicon International, CA, USA) were diluted at 1/200 in phosphate buffered saline (Tomofuji *et al*., 2006). The color was developed with 3-3'-diamino benzidine tetrahydrochloride. To identify osteoclasts, TRAP activity was detected by using the azo dye method (Endo *et al*., 2013). Sections were counterstained with Mayer's hematoxylin.

A blinded examiner (T.T.) performed the following histometric analyses with a light microscope. The distance between the cemento-enamel junction and the alveolar bone crest (a marker of alveolar bone loss) was measured with a microgrid at a magnification of ×200 (Irie *et al*., 2008). The numbers of 8-OHdG–positive cells and total cells in standard areas (0.1 mm^2 each) adjacent to the cementum (two serial areas from the top of the cementum) were determined (Tomofuji *et al*., 2006). TRAP-positive osteoclasts on the surface of alveolar bone were counted by using a light microscope at ×400 magnification and reported as number/millimeter (Endo *et al*., 2013). We evaluated intra-examiner reproducibility by double-scoring 10 randomly selected sections at 2-week intervals. The agreement with one 8-OHdG–positive cell was more than 80%.

Real-time RT-PCR

Total RNA was isolated from the gingival biopsy samples by using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The isolated RNA was quantified by measuring the absorbance at 260 nm, and the purity was determined by the 260/280 nm absorbance ratio. Only samples with a ratio of >1.8 were used (Endo *et al*. 2013). Total RNA was reverse-transcribed by using AMV Reverse Transcriptase (Takara Bio Inc., Shiga, Japan) at 42°C for 30 minutes. Real-time PCR was performed by using SYBR Green Realtime PCR Master Mix (Toyobo, Osaka, Japan) in a real-time QPCR system (Agilent Technologies, Tokyo, Japan). The primer sequences of IL-1β, NLRP3, caspase-1, apoptosis-associated speck-like protein (ASC), nuclear factor (NF)-κB, and β-actin are shown in Table 1.

The amplification conditions were as follows: 40 cycles at 95°C (30s), 67°C (30 s), 72°C (20 s) for IL-1β, NLRP3, caspase-1 and ASC; 45 cycles at 95°C (15s), 54°C (20s), 72°C (20 s) for NF-κB; and 45 cycles at 95°C (10 s), 54°C (20 s), 72°C (10 s) for β-actin. The mRNA levels were calculated in terms of the relative copy number ratio of each mRNA to β-actin for each sample.

Measurements of Serum 8-OHdG and CoQ10

The level of 8-OHdG was determined using the ELISA kit (JaICA, Shizuoka, Japan). In

addition, serum levels of total CoQ10 (rCoQ10 and oxidized form of CoQ10) were

quantified by high-performance liquid chromatography (Kunitomo *et al*., 2008).

Statistical analysis

The data are presented as mean values ± standard deviation (SD). The differences in

histological data and serum ROM level among the four groups were analyzed by one way

ANOVA followed by Tukey's method. The *T*-test was used for statistical comparisons of

gene expression, 8-OHdG positive ratio, TRAP-positive osteoclasts and serum CoQ10

level between the control and the experimental groups. P <0.05 was considered

statistically significant.

RESULTS

The body weights (mean ± SD) of the rats in the 2-month-old (n=6), 4-month-old (n=6), and 6-month-old (n=12) groups were 207 ± 9 g, 265 ± 8 g, and 324 ± 28 g, respectively. There were no significant differences between the control and experimental groups in terms of food consumption, body weight or growth pattern during the experimental period.

The linear distances between the cemento-enamel junction and alveolar bone crest (mean ± SD) of the rats in the 2-month-old (n=6), 4-month-old (n=6), and 6-month-old (n=12) groups were 497 ± 45 μm, 561 ± 33 μm, and 601 ± 36 μm, respectively, and these values were significantly higher in the 6-month-old rats than in the 2-month-old rats ($p< 0.05$). On the other hand, there were no significant differences between the control and experimental groups in terms of the linear distance between the cemento-enamel junction and alveolar bone crest at 6 months of age.

At 6 months, both the control and experimental groups showed no pathological changes such as extension of blood vessels or increased number of inflammatory cells. However, the periodontium in the experimental group exhibited low expression levels of 8-OHdG and TRAP, as compared to the control group (Fig. 1). The ratio of 8-OHdG-positive cells and the number of TRAP-positive osteoclasts in the experimental group were significantly lower than those in the control group ($p< 0.05$).

Gene expression of NLRP3, caspase-1, ASC and IL-1β in periodontal tissues was significantly lower in the experimental group as compared to the control group ($p < 0.05$) (Fig. 2). Gene expression of NF-κB in periodontal tissues was also lower in the experimental group than in the control group ($p < 0.05$).

Serum levels of 8-OHdG tended to increase in an age-dependent manner, and these values were significantly lower in the experimental group than in the control group at 6 months of age ($p < 0.05$) (Fig. 3). In addition, serum levels of CoQ10 (mean ± SD) at 6 months of age were 0.018 ± 0.002 µg/ml in the control group and 0.023 ± 0.002 µg/ml in the experimental group. There was a significant difference in the serum total CoQ10 levels of the control group and the experimental group ($p < 0.05$).

DISCUSSION

To the best of our knowledge, this is the first study demonstrating the anti-aging effects

of rCoQ10 on periodontal tissue in rats. In this study, the serum level of 8-OHdG

increased with age, indicating that aging induced oxidative stress in our model. Topically

applied rCoQ10 decreased the serum level of 8-OHdG at 6 months of age. Furthermore,

lower expression of 8-OHdG, a parameter of oxidative DNA damage (Tomofuji *et al*.,

2006), was found in the rCoQ10-treated rats as compared to the control rats at 6 months.

These observations indicate that rCoQ10 could reduce oxidative stress during aging, and

this effect might suppress age-related oxidative stress in periodontal tissue.

The concentration of CoQ10 in rat skin increased when a solution of 1% CoQ10 in

olive oil was topically applied (Giovannini *et al*., 1988). Therefore, in the present study,

we used a 1% concentration of rCoQ10 in ointment. In addition, the serum level of total

CoQ10 was higher in the rCoQ10-treated group than in the control group. In our model,

the applied ointment was wiped off to avoid swallowing. These results show that rCoQ10

penetrated the periodontal tissue in our model.

Oxidative stress induces inflammatory reactions by activating the NLRP3

inflammasome (Kauppinen *et al*., 2012; Salminen *et al*., 2012). In the present study,

rCoQ10 suppressed the expression of genes that encode the NLRP3 inflammasome, such

as NLRP3, ASC, and Caspase-1, in the periodontal tissue. Furthermore, the rCoQ10-treated group had a lower level of IL-1β expression than the control group. It is feasible that the anti-oxidative effects of rCoQ10 could prevent inflammatory reactions by inactivation of the NLRP3 inflammasome.

Our observations also revealed that rCoQ10 down-regulated the gene expression of NF-κB. NF-κB is involved in oxidative stress–induced inflammation, including IL-1β expression (Gloire *et al*., 2006). Therefore, it is also possible that rCoQ10 inhibits inflammatory reactions by inactivation of NF-κB.

IL-1β is a chemical mediator of osteoclast differentiation (Bloemen *et al*., 2011). In the present study, rCoQ10 decreased the number of TRAP-positive osteoclasts on the surface of alveolar bone. This result indicates that rCoQ10 ameliorates the effects of aging on osteoclast differentiation by reducing the expression of IL-1β. On the other hand, our observations showed no differences in the distance between the cemento-enamel junction and the alveolar bone crest with or without rCoQ10 application. In the present study, the experimental period was set as two months to reduce the influence of general anesthesia on periodontal tissues (Ekuni *et al*., 2008). However, a longer period may be necessary to observe a rCoQ10-induced change in alveolar bone level.

CoQ10 applied topically to human skin for 6 months reduces the depth of wrinkles

(Hoppe *et al*., 1999), and CoQ10 intake for 6 weeks improved age-related impairment in spatial performance in mice (Shetty *et al*., 2013). These observations are consistent with the current results suggesting that rCoQ10 has anti-aging properties at a periodontal tissue level.

The effects of CoQ10 on periodontal tissue have been studied previously. A clinical study suggested that topical application of CoQ10 improved chronic periodontitis not only as a sole treatment but also in combination with traditional nonsurgical periodontal treatment (Hanioka *et al*., 1994). It was also suggested that rCoQ10 serves as an endogenous antioxidant, which increases the concentration of CoQ10 in inflamed gingiva and effectively suppresses advanced periodontitis (Prakash *et al*., 2010). These reports demonstrate the effect of CoQ10 on periodontal inflammation. On the other hand, our findings indicate the anti-aging effect of rCoQ10 on normal periodontal tissue, which is the novel aspect of the present study.

This study has some limitations. Initially, it was shown that there is a dose-response relationship between the amount of CoQ10 applied and the CoQ10 skin concentration (Giovannini *et al*., 1988). Therefore, the optimal dose of rCoQ10 required for efficacy against periodontal aging should be determined with the current model. We started the topical application of rCoQ10 at 4 months, by which time the rats had reached adulthood

(Arai *et al*., 2005). In the future, it may be necessary to clarify how topically applied

rCoQ10 modulates the effects of aging on periodontal tissue in older rats.

In conclusion, topically applied rCoQ10 could diminish oxidative stress, which in

turn might suppress age-related inflammatory reactions in periodontal tissue.

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REFERENCES

Arai K, Tanaka S, Yamamoto-Sawamura T, Sone K, Miyaishi O, Sumi Y (2005). Aging changes in the periodontal bone of F344/N rat. *Arch Gerontol Geriatr* 40: 225-229.

Blatt T, Littarru GP (2011). Biochemical rationale and experimental data on the antiaging properties of CoQ(10) at skin level. *Biofactors* 37: 381-385.

Bloemen V, Schoenmaker T, de Vries TJ, Everts V (2011). IL-1β favors osteoclastogenesis via supporting human periodontal ligament fibroblasts. *J Cell Biochem* 112: 1890-1897.

Chung HY, Lee EK, Choi YJ, Kim JM, Kim DH, Zou Y, *et al*. (2011). Molecular inflammation as an underlying mechanism of the aging process and age-related diseases. *J Dent Res* 90: 830-840.

Ekuni D, Tomofuji T, Tamaki N, Sanbe T, Azuma T, Yamanaka R, *et al*. (2008). Mechanical stimulation of gingiva reduces plasma 8-OHdG level in rat periodontitis. *Arch Oral Biol* 53: 324-329.

Endo Y, Tomofuji T, Ekuni D, Azuma T, Irie K, Kasuyama K, *et al*. (2013). Preventive effects of trehalose on osteoclast differentiation in rat periodontitis model. *J Clin Periodontol* 40: 33-40.

Giovannini L, Bertelli AA, Scalori V, Dell'Osso L, Alessandrì MG, Mian M (1988). Skin

penetration of CoQ10 in the rat. *Int J Tissue React* 10: 103-105.

Gloire G, Legrand-Poels S, Piette J (2006). NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem Pharmacol* 72: 1493-1505.

Hanioka T, Tanaka M, Ojima M, Shizukuishi S, Folkers K (1994). Effect of topical application of coenzyme Q10 on adult periodontitis. *Mol Aspects Med* 15 Suppl: s241-s248.

Hoppe U, Bergemann J, Diembeck W, Ennen J, Gohla S, Harris I, *et al*. (1999). Coenzyme Q10, a cutaneous antioxidant and energizer. *Biofactors* 9: 371-378.

Irie K, Tomofuji T, Tamaki N, Sanbe T, Ekuni D, Azuma T, *et al*. (2008). Effects of ethanol consumption on periodontal inflammation in rats. *J Dent Res* 87: 456-460.

Kauppinen A, Niskanen H, Suuronen T, Kinnunen K, Salminen A, Kaarniranta K (2012). Oxidative stress activates NLRP3 inflammasomes in ARPE-19 cells--implications for age-related macular degeneration (AMD). *Immunol Lett* 147: 29-33.

Kunitomo M, Yamaguchi Y, Kagota S, Otsubo K (2008). Beneficial effect of coenzyme Q10 on increased oxidative and nitrative stress and inflammation and individual metabolic components developing in a rat model of metabolic syndrome. *J Pharmacol Sci* 107: 128-137.

Littarru GP, Tiano L (2007). Bioenergetic and antioxidant properties of coenzyme Q10:

recent developments. *Mol Biotechnol* 37: 31-37.

Prakash S, Sunitha J, Hans M (2010). Role of coenzyme Q(10) as an antioxidant and bioenergizer in periodontal diseases. *Indian J Pharmacol* 42: 334-337.

Rai B, Kaur J, Catalina M, Anand SC, Jacobs R, Teughels W (2011). Effect of simulated microgravity on salivary and serum oxidants, antioxidants, and periodontal status. *J Periodontol* 82: 1478-1482.

Ryo K, Ito A, Takatori R, Tai Y, Arikawa K, Seido T, *et al*. (2011). Effects of coenzyme Q10 on salivary secretion. *Clin Biochem* 44: 669-674.

Salminen A, Ojala J, Kaarniranta K, Kauppinen A (2012). Mitochondrial dysfunction and oxidative stress activate inflammasomes: impact on the aging process and age-related diseases. *Cell Mol Life Sci* 69: 2999-3013.

Shetty RA, Forster MJ, Sumien N (2013). Coenzyme Q(10) supplementation reverses age-related impairments in spatial learning and lowers protein oxidation. *Age (Dordr)*: in press.

Takane M, Sugano N, Iwasaki H, Iwano Y, Shimizu N, Ito K (2002). New biomarker evidence of oxidative DNA damage in whole saliva from clinically healthy and periodontally diseased individuals. *J Periodontol* 73: 551-554.

Tomofuji T, Azuma T, Kusano H, Sanbe T, Ekuni D, Tamaki N, *et al*. (2006). Oxidative

damage of periodontal tissue in the rat periodontitis model: effects of a high-cholesterol

diet. *FEBS Lett* 580: 3601-3604.

Figure 1. Photographs (original magnification× 20; 8-OHdG [A and B] and TRAP [C and

D]) of rat periodontal tissue. The experimental group (B and D) showed 8-OHdG positive

cells (brown-stained nuclei) and TRAP-positive osteoclasts (red-stained cytoplasm) less

than the control group (A and C). In addition, there were significant differences between

the two groups with regard to the ratio of 8-OHdG positive cells to total cells (E) and

number of TRAP-positive osteoclasts (F). AB, alveolar bone, CM, cementum, GCT,

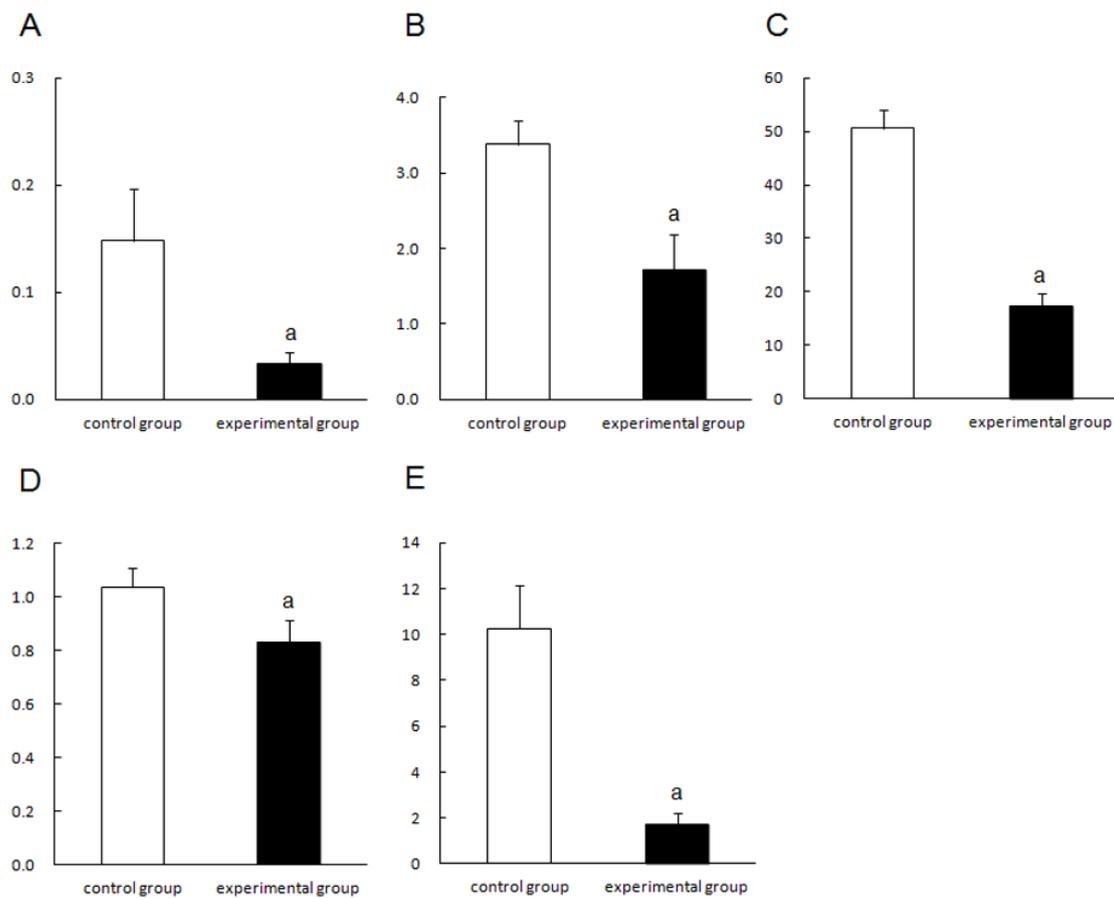gingival connective tissue and JE, junctional epithelium.

[a] $p< 0.05$, compared to the control group.
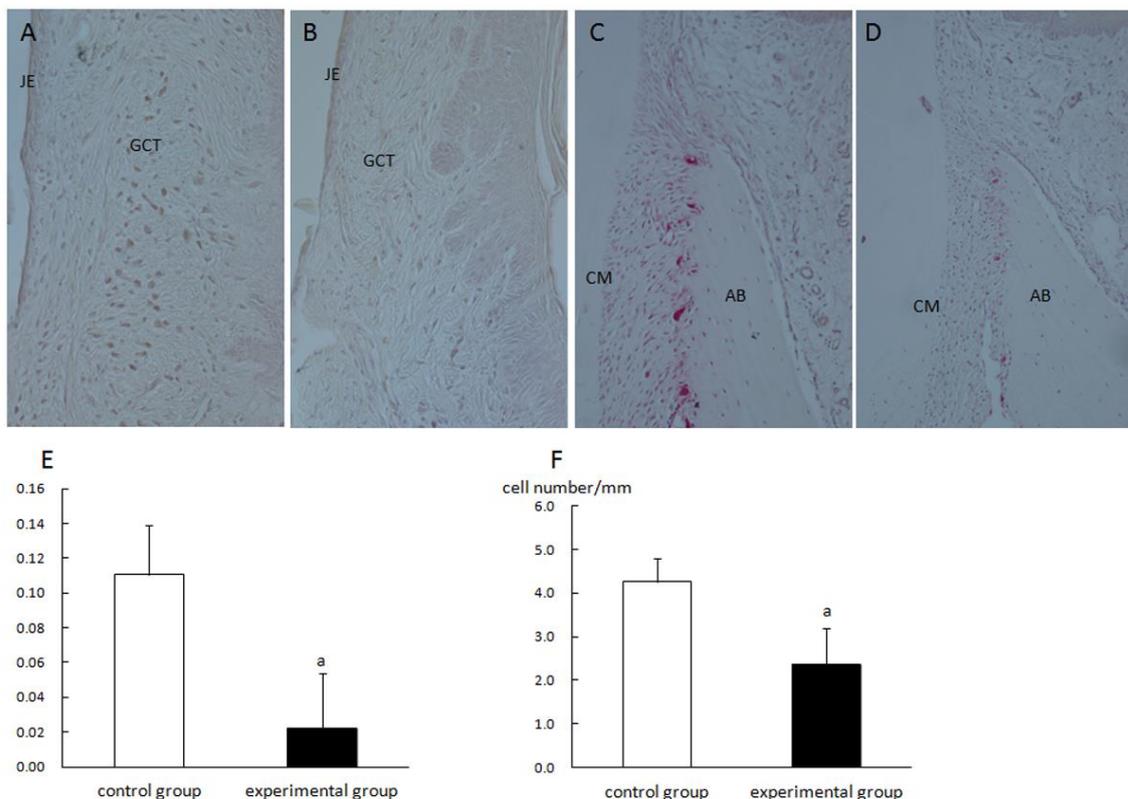
Figure 2. Gene Expression for NALP3 (A), Caspase-1 (B), ASC (C), IL-1β (D) and NF-κB (E) in rat periodontal tissue.

Bars represent mean ± SD of 6 rats.
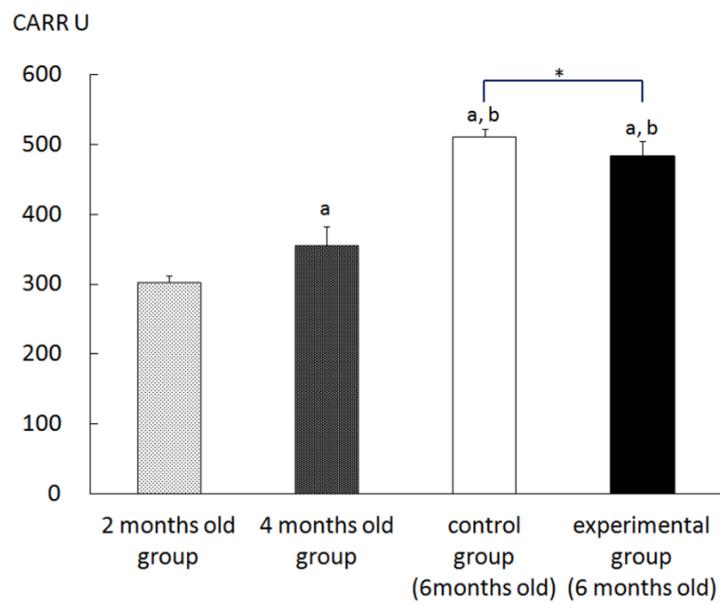
[a] $p < 0.05$, compared to the control group.

Figure 3. Serum levels of 8-OHdG in Rats.

Bars represent mean ± SD of 6 rats.

[a] $p < 0.05$, compared to the 2-months old group.

[*] $p < 0.05$, compared to the control group (6-months old).

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Table 1. Primer sequences for NALP3 inflammasomes.

	Sense (5′–3′)	Antisense (5′–3′)	Length (bp)	Accession no.
NALP3	GCTGCTCAGCTCTGACCTCT	AGGTGAGGCTGCAGTTGTCT	165	NM_001191642.1
Caspase-1	TGCCTGGTCTTGTGACTTGGAG	ATGTCCTGGGAAGAGGTAGAAACG	133	NM_172322.1
ASC	TTATGGAAGAGTCTGGAGCTGTGG	AATGAGTGCTTGCCTGTGTTGG	101	NM_012762.2
IL-1β	CACCTCTCAAGCAGAGCACAGA	ACGGGTTCCATGGTGAAGTC	81	NM_031512.2
NFκB	CACTCTCTTTTTGGAGGT	TGGATATAAGGCTTTACG	206	NM_199267.2
β-actin	TGTTGCCCTAGACTTCGAGCA	GGACCCAGGAAGGAAGGCT	155	NM007393