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Original Article

Therapeutic effects of caffeic acid phenethyl ester on alveolar bone loss in rats with endotoxin-induced periodontitis

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Abstract *Background/purpose:* Caffeic acid phenethyl ester (CAPE) is an antioxidant which is decreases the bone resorption and enhances the bone healing. The aim of this study was to investigate the effects of administering systemic CAPE on alveolar bone loss in rats with experimental periodontitis.
Materials and methods: Thirty male Sprague Dawley rats were divided into three groups: control, endotoxin-induced periodontitis (EP), and EP treated with CAPE (EP-CAPE). Endotoxin was injected into the gingiva of test rats on days 1, 3, and 5, whereas saline was injected into the control rats. The EP-CAPE group received 10 mmol/kg/day CAPE intraperitoneally for 28 consecutive days. Saline was given in the control and EP groups in the same manner. At the end of the study, intracardiac blood samples were obtained, and the rats were sacrificed. Alveolar bone loss was analyzed with histometric measurements. The oxidative stress index (OSI) was used to evaluate the oxidative stress. The receptor activator of the nuclear factor kappa B ligand (RANKL) level was analyzed stereologically.
Results: CAPE administration significantly decreased the serum OSI and interleukin-1β levels. Alveolar bone loss was statistically higher in the EP group compared with the EP-CAPE group

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($P < 0.05$). Immunohistochemical analyses of the RANKL were significantly lower in the EP-CAPE group than in the EP group ($P < 0.05$).
Conclusion: This experimental study revealed that CAPE administration significantly prevented alveolar bone loss and stimulated periodontal tissue healing.

Introduction

Periodontitis is an infectious disease characterized by gingival inflammation and the destruction of the periodontal tissues.[1] Environmental and genetic factors and interaction between the host immune response and specific bacteria affect periodontal destruction.[2] An increase in inflammatory cytokines and the impaired balance of the osteoclast and osteoblast levels are major reasons for inflammation-induced alveolar bone resorption in periodontitis.[3] Osteoclasts are the primary cells responsible for bone loss. Inflammatory cytokines control the osteoclastogenesis via receptor activator of nuclear factor-kappa B (RANK) and its ligand (RANKL), interleukin (IL)- 1, interleukin-6, tumor necrosis factor alpha, and prostaglandin E2.[3] RANKL is a members of the tumor necrosis factor superfamily and is expressed by osteoblasts, stromal cells, and other mesenchymal cells. Osteoprotegerin (OPG) is expressed by periodontal tissue cells and binds to RANKL. RANKL and OPG play an important role in the differentiation, activation, and survival of osteoclast precursors and osteoclasts and in regulating bone remodeling.[4,5] The RANKL/OPG ratio increases in individuals with periodontitis.[6]

Reactive oxygen species (ROS) are produced by human cells and are necessary for normal metabolic activity. A balance exists between the ROS levels and antioxidant defense activity in the normal cellular process. When this balance changes in favor of ROS by increasing ROS levels or decreasing antioxidant defense, oxidative stress can result and lead to breakdown in periodontal tissues.[7] In addition, ROS induce the RANKL levels and play a critical role in the formation and survival of osteoclast cells.[8] Furthermore, they can initiate lipid peroxidation, damage the DNA and protein, and stimulate inflammatory cytokines such as IL-1β.[9]

Caffeic acid phenethyl ester (CAPE), a lipid-soluble active compound derived from the propolis of honey bees, is an antioxidant. It has been reported that CAPE has anti-inflammatory, immunomodulatory, anticancer properties.[10–12] Additionally, CAPE accelerates wound healing, reduces RANKL-induced osteoclastogenesis, and decreases the tissue destruction caused by oxidative stress.[13–15] It also stimulates bone healing.[16–18] However, the effect of CAPE on periodontal disease has not been examined in animals. The purpose of this study was to investigate the effects of CAPE on alveolar bone loss in rats with experimental periodontitis by using biochemical and immunohistochemical analysis.

Materials and methods

Animals

All procedures were evaluated and approved by the Pamukkale University Ethics Committee for Animal Experimentation (PAUHADYEK-2016/8). Thirty male, three-month old Sprague Dawley rats with a mean weight of between 220 and 250 g were used in this study. The animals were randomized into three groups: control, rats with endotoxin-induced periodontitis (EP), and rats with periodontitis treated with CAPE (EP-CAPE). All of the animals were housed in temperature-controlled rooms and given standard rat food and water ad libitum.

Experimental periodontitis induction

An endotoxin procedure was performed to induce experimental periodontitis in the rats. 10 μL of *Escherichia coli* E (Serotype 055: B5, L2637; Sigma Chemical Co., St. Louis, MO, USA; 1 mg/mL) was injected into the vestibular gingival sites between the right first and second maxillary molars.[19] The endotoxin was injected under anesthesia (Xylazine hydrochloride-10 mg/kg, Ketamine hydrochloride- 40 mg/kg) on days 1, 3, and 5. The control rats received saline in the same way.

CAPE administration

In this study, CAPE (Sigma, St. Louis, MO) was dissolved in absolute ethanol and diluted with saline. After the endotoxin injection, a daily 10 mmol/kg dose of CAPE was administered intraperitoneally (ip) in the EP-CAPE group for 28 day according to previously described studies.[16] CAPE was administered at the same time every day for standardization. The control and EP groups received saline in the same dosage and at the same times ip.

Blood and tissue sample collection

On day 28, all animals were anesthetized, blood samples were collected from their hearts by puncture, and the animals were decapitated. Cardiac blood samples were centrifuged and serum samples were frozen at −80 °C for biochemical assay. The right maxillae of the rats were removed and fixed with a 10% neutral formaldehyde solution for histological analyses.

Serum interleukin-1β assay

Concentrations of interleukin-1β (IL-1β) were evaluated by rat-specific enzyme-linked immunoassay (ELISA) kit (Fine Biotechnology, Wuhan, China), according to the manufacturer's instructions.

Serum C-terminal telopeptide of type I collagen assay

Serum C-terminal telopeptide of type I collagen (CTX) concentrations were determined using a rat-specific ELISA kit (Fine Biotechnology, Wuhan, China), according to the manufacturer's instructions.

Evaluation of oxidative stress

Serum total antioxidant status (TAS) and total oxidant status (TOS) levels were determined using relevant available ELISA kits (Rel Assay Diagnostics, Gaziantep, Turkey), according to the manufacturer's instructions. The results of TAS were defined as millimolar Trolox equivalent per liter (mmol Trolox Eq/L protein). The results of TOS were defined as micromolar hydrogen peroxide equivalent per liter (mmol H_2O_2 Eq/L protein). The oxidative stress index (OSI) was calculated as the percentage ratio of TOS to TAS, according to a previously described study.[20]

Histological imaging

After fixation of the maxillary tissues for 72 h, tissue samples were incubated in 6% nitric acid solution for decalcification over one week. Solution was re-added every day and decalcification was assessed by needle in the last few days. After the tissues had decalcified, they were dehydrated in alcohol, embedded in paraffin wax, and sectioned buccolingually with a microtome (Leica RM2125RT, Leica Instruments, Nubloch, Germany). The obtained sections were stained with Crossman-modified Mallory triple, and photographs were taken using a light microscope with a camera attachment (Nikon Eclipse i50; Nikon, Tokyo, Japan), as previously described.[21]

Immunohistochemical analyses

The sections (5 μm thickness) were stained with anti-RANKL kit (Santa Cruz Biotechnology, Santa Cruz, C.) (1:50 dilutiona) for immunohistochemical assay. The binding area of the antibodies was assessed with a high-power light microscope (Nikon Eclipse i50; Nikon, Tokyo, Japan). The number of RANKL-positive cells in 10 sections of alveolar bone for each rat was calculated using a stereologic optical fractionator method (Fig. 1). Stereologic analyses were applied using a stereology workstation consisting of stereology software (Stereo-Investigator, v.9.0, Microbrightfield, Williston, VT.) and a modified light microscope (Leica DM4000B, Leica Instruments). To calculate the periodontal bone support, the distance among the root apex and epithelial attachment was divided the distance among the root apex and crown tip.

Statistical analyses

One-way ANOVA and the Duncan post hoc test were used with statistical software (SPSS v.17.0, IBM, Chicago, IL.) for statistical analyses in this study. All data are reported as mean ± SD for each group ($\alpha = 0.05$).

Results

Biochemical results

The biochemical results for all groups are shown in Fig. 2. The serum CTX level in the EP group was significantly higher than in the control group ($P < 0.05$). However, CAPE treatment did not significantly decrease the serum CTX level in the EP-CAPE group compared with the EP group ($P > 0.05$). The serum IL-1β level in the control group was significantly lower than in the disease groups ($P < 0.05$), and the serum IL-1β level was also significantly lower in the EP-CAPE group with respect to the EP group ($P < 0.05$). The serum OSI level increased significantly in the EP group compared with the control group ($P < 0.05$). Additionally, the CAPE treatment decreased significantly in the OSI level in the EP-CAPE group compared with the EP group ($P < 0.05$).

Histologic results

In the control group, periodontal tissues showed normal tissue structure. Histologic findings revealed increased destruction of the periodontal ligament, alveolar bone loss, and cemento-enamel attachment loss in the EP group (Fig. 3). However, CAPE administration decreased bone resorption, attachment loss, and damage to the periodontal ligament. RANKL-positive osteoclast cell density was found to be significantly higher in the EP group than in the control group ($P < 0.05$). Also, the CAPE treatment significantly

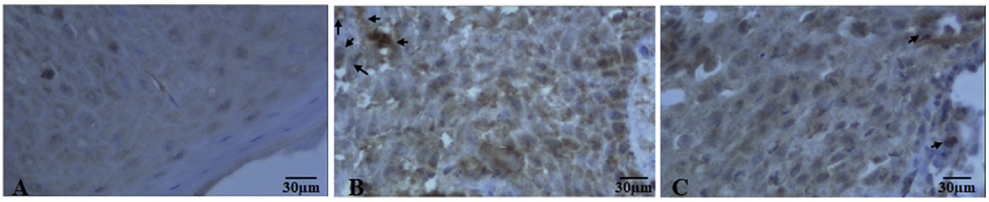

Figure 1 The numerical density values of anti-RANKL-positive osteoclasts. A) Control group B) EP group C) EP-CAPE group. The arrows indicate anti-RANKL-positive osteoclasts.

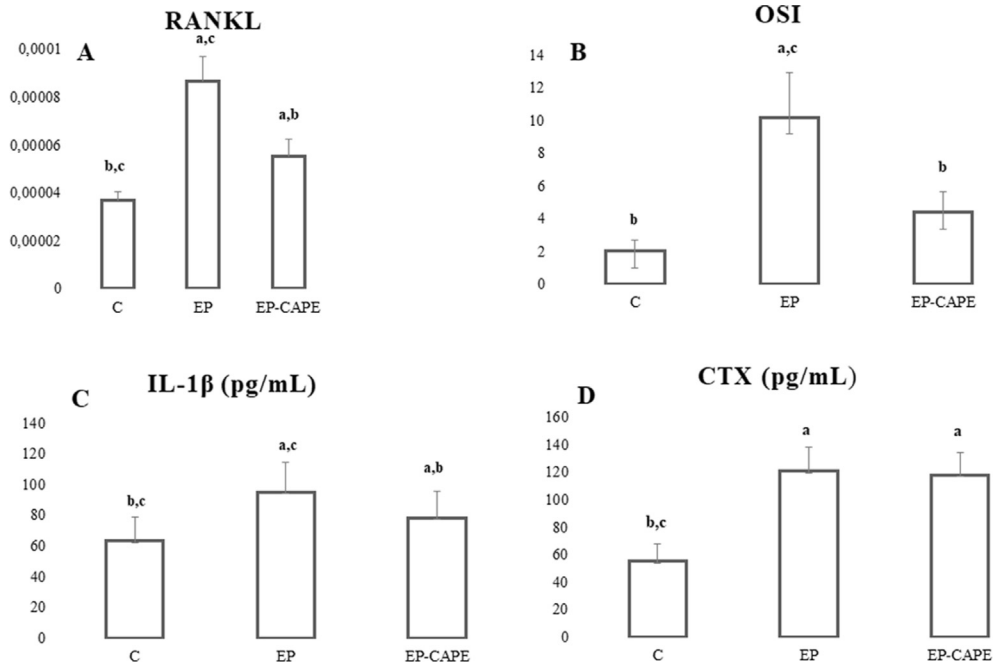

Figure 2 Comparison of biochemical and immunohistochemical results. A) RANKL B) OSI C) IL-1β D) CTX. The values were expressed as means ± SD. a Statistically significant difference ($P < 0.05$) according to control group. b Statistically significant difference ($P < 0.05$) according to EP group. c Statistically significant difference ($P < 0.05$) according to EP-CAPE group.

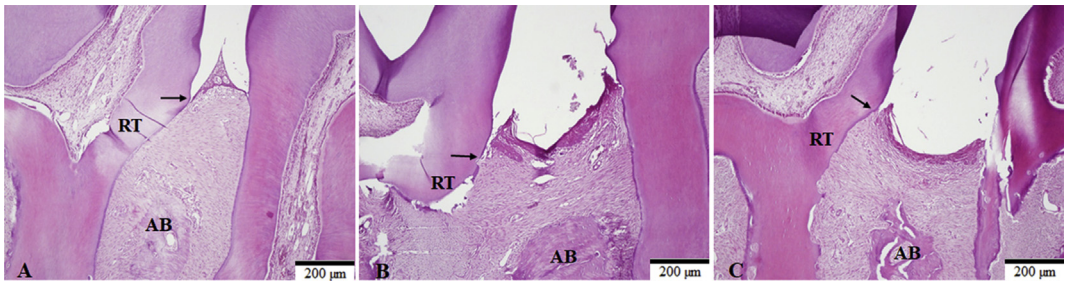

Figure 3 Histologic view of gingival mucosal tissues from all groups for maxillar first molar tooth. A) Control group section B) EP group section C) EP-CAPE group section. AB = Alveolar bone; RT = Root of tooth. The cemento-enamel junction is indicated by the arrowheads.

decreased these osteoclast cells compared with the EP group ($P < 0.05$) (Fig. 2).

MPBS and DPBS

The comparison of the mean values of MPBS and DPBS proportions among all groups are shown in Fig. 4. The values of MPBS and DPBS were significantly higher in the control group compared with the disease groups ($P < 0.05$). However, these values in the CAPE treatment group were significantly higher compared with those of the EP group ($P < 0.05$).

Discussion

In this study, the therapeutic efficacy of CAPE was investigated in rats with endotoxin-induced experimental periodontitis. The injection of endotoxin induced periodontal destruction and alveolar bone loss in rats.[19,22–24] The present study indicated that the endotoxin injection increased the alveolar bone loss and confirmed previous studies. Our study, however, found that CAPE decreased endotoxin-induced bone loss, OSI levels, and RANKL levels.

CAPE has an antioxidant efficacy. Several studies have indicated that it improves the bone healing process, prevents RANKL-induced osteoclastogenesis, and may be used as a regenerative agent in the therapy of bone resorptions.[14,16–18] Previous studies have reported that antioxidant therapy enhanced bone healing in periodontitis.[21,25–27] However, studies that determined the efficacy of CAPE in periodontitis are lacking. To our knowledge, this is the first study in which the effect of CAPE on alveolar bone loss has been investigated.

Application techniques, dosage, and periods of CAPE are important for new bone formation. Local application of 100 mmol/kg CAPE is more effective than 50 mmol/kg CAPE and systemic injection of CAPE is more beneficial than local

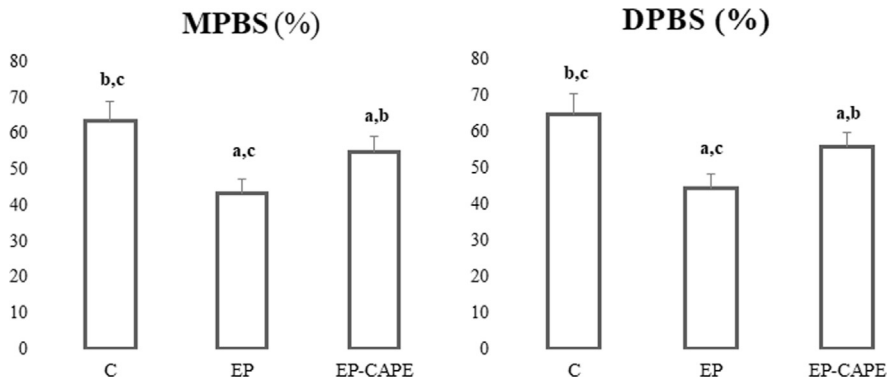

Figure 4 Comparison of MPBS and DPBS percentages. The values were expressed as means ± SD. a Statistically significant difference ($P < 0.05$) according to control group. b Statistically significant difference ($P < 0.05$) according to EP group. c Statistically significant difference ($P < 0.05$) according to EP-CAPE group.

application with regard to bone healing. It has also been demonstrated that a systemic injection of 10 mmol/kg CAPE significantly increased new bone formation in 28 days.[16] Hence, we administered 10 mmol/kg CAPE systemically for 28 days according to previous study.[16]

In vitro study, Cheng et al. reported that *Escherichia coli* lipopolysaccharide (LPS) increased the ROS level.[28] Increased of the ROS level causes the periodontal destruction by elevating the oxidative stress.[7] OSI is expressed as the TOS/TAS ratio and is used to evaluate the oxidative stress. Several studies have indicated an association of periodontitis with the TOS/TAS ratio.[20,29] It has been reported that there is a correlation between low TAS levels and periodontal status.[30,31] Tomofuji et al. applied the LPS/protease topically in gingival sulcus for induce the periodontitis and they reported that periodontitis stimulates the circulating oxidative stress.[32] In addition, Kose et al. showed that periodontal destruction elevates the serum OSI level in the rats with periodontitis.[27] Serarslan et al. stated that CAPE decreased oxidative stress.[33] The present study shows that OSI significantly increased in the serum of the EP group compared with the control. These results are consistent with previous studies.[27,30–32] In addition, serum decreased of OSI levels in the EP-CAPE group compared with the EP group supported the fact that CAPE has an antioxidant effect and decreases the oxidative stress on tissue.[13–15]

Osteoclastic bone resorption is related to elevation of the RANKL tissue level. The increase in the RANKL level causes alveolar bone destruction in periodontitis.[34] Some studies have demonstrated that antioxidant agents decreased the RANKL levels and inhibited the RANKL-induced osteoclastogenesis.[21,35] To analyze alveolar bone loss, the present study evaluated the activation of RANKL. Histological analyses revealed lower RANKL levels in the EP-CAPE group than in the EP group, and these findings are consistent with previous studies.[15] The significant decrease in RANKL in the EP-CAPE group may be related to the anti-osteoclastic effect of CAPE by suppressing the RANKL-induced osteoclastogenesis, thus inhibiting alveolar bone loss. As a result, these findings confirmed that CAPE improved bone healing.[16–18]

CTX is considered a specific marker of bone resorption and is commonly used to evaluate bone disorders. One study reported that decreasing serum CTX prevented osteoclastic activity.[36] In this study, periodontitis increased the serum CTX levels, and these findings supported those of previous studies.[21,37] Several studies have indicated that CAPE does not reduce the serum level of CTX.[38,39] The present results demonstrate that CAPE administration does not have a positive effect on serum CTX levels in periodontitis, and these findings are consistent with previous studies.[38,39]

Injection of LPS stimulates cytokine synthesis in the periodontal tissues, and this process causes tissue destruction.[40] It has been indicated that LPS induced release of IL-1β, IL-6 and IL-8 cytokines.[41] The present study showed that LPS increased levels of IL-1β in the EP and EP-CAPE groups. These results corroborate the fact that the injection of LPS stimulates the release of IL-1β. In the present study, IL-1β levels were found significantly higher in the EP group than EP-CAPE groups, and these results confirmed previous studies that reported an anti-inflammatory effect and modulation of the immunological processes by CAPE.[10,11]

Immunohistochemical measurement revealed higher alveolar bone loss in the EP group compared with other groups. The analysis of MPBS and DPBS showed that systemic CAPE administration limited alveolar bone loss. The current findings verified that the CAPE administration contributes to bone healing by decreasing the oxidative stress and RANKL levels and are consistent with a previous studies.[13–18]

In conclusion, to the best of our knowledge, this is the first study to examine the therapeutic effect of CAPE on endotoxin-induced periodontitis. The data obtained from this study demonstrate that administration of CAPE inhibits the alveolar bone loss in periodontitis by decreasing the serum RANKL levels. Therefore, CAPE may be used as a therapeutic agent for the treatment of periodontal disease. However, the therapeutic effect of CAPE should be verified by further studies.

Conflicts of interest

The authors report no conflicts of interest related to this study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jds.2019.03.011.

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