



EVALUATION OF CAFFEIC ACID PHENETHYL ESTER ADMINISTRATION IN CHRONICALLY STRESSED RATS WITH EXPERIMENTAL PERIODONTITIS

ABSTRACT

Objectives: The aim of the present study was to investigate the therapeutic effects of systemic caffeic acid phenethyl ester treatment on oxidative stress and alveolar bone destruction in lipopolysaccharide (LPS)-induced periodontitis (EP) group in chronically stressed rats.

Materials and Methods: Forty male Sprague Dawley rats (EP-CS-CAPE) group were divided into four groups: 1) control group, 2) experimental periodontitis (EP), 3) EP and chronic stress (CS) group (EP-CS), and 4) EP-CS treated with CAPE (EP-CS-CAPE). To induce periodontitis, LPS was administered into the buccal gingiva of the test groups, and pure saline was administered for the interleukin (IL)-1 β levels were control group. Two test groups were exposed to restraint stress and one group of these groups was treated with only a single dose of CAPE (10 mmol/kg). Likewise, saline was administered in the control, EP, and EP-CS groups. After 14 days, serum samples were collected from the heart, and all rats were sacrificed for analyses. Oxidative stress and interleukin (IL)-1 β were investigated. The receptor activator of the nuclear factor kappa B ligand (RANKL) and alveolar bone loss were determined by immunohistochemical analysis.

Results: The oxidative stress, alveolar bone loss, IL-1 β and RANKL levels were found significantly higher in the EP-CS group compared with control and EP groups ($p<0.05$). However, the administration of RANKL level CAPE significantly reduced oxidative stress and IL-1 β in the EP-CS-CAPE group compared with the EP-CS group ($p<0.05$). Also, CAPE treatment significantly reduced RANKL and alveolar bone loss in the EP-CS-CAPE group compared with the EP-CS group ($p<0.05$).

Conclusions: The present results indicated that CAPE may inhibit alveolar bone loss by modulating the immune response and inflammatory process.

Keywords: Alveolar bone loss, antioxidants, periodontitis

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INTRODUCTION

Inflammatory periodontitis leads to the destruction of tooth-supporting tissues and to alveolar bone loss. Factors including genetic predisposition, environmental factors, and the association between pathogenic bacteria and the host immune response increase the severity and progression of periodontitis.¹ In recent years, psychologic factors, such as anxiety, stress, and depression, have been suggested as playing a critical role in periodontal disease.^{2,3} Studies have reported that stress accelerates alveolar bone resorption and periodontal attachment loss and impairs tissue wound healing.^{4,5}

Stress can activate the hypothalamic–pituitary–adrenal (HPA) axis, and activation of the HPA axis stimulates glucocorticoid hormones from the adrenal cortex.⁶⁻⁸ These corticoid hormones increase proinflammatory cytokines levels.⁹ Additionally, activation of the HPA axis elevates the brain corticosterone level, and this corticosterone stimulates the overproduction of glutamate in the cortical and limbic areas.¹⁰ The stimulation of glutamate release enhances the metabolic rate by disrupting mitochondrial activity.¹¹ The increased metabolic rate causes breakdown of the balance between the anti-oxidant defense system and the reactive oxygen species (ROS) level by overproduction of free radicals.¹² Furthermore, especially in chronic stress (CS)-depressed humans, the immune response is altered by reducing immunoglobulin A and G, impairing neutrophil activity, and suppressing cytokine production.¹³

CAPE is an antioxidant and has anti-inflammatory and antitumoral effects.¹⁴ Gremy *et al.* reported that CAPE regulated the immune response by preventing the apoptosis.¹⁵ Recent reports have suggested that CAPE improves tissue wound healing, avoids osteoclastogenesis by induced the receptor activator of the nuclear factor kappa B ligand (RANKL), decreases ROS and oxidative stress, and contributes to bone healing.^{14, 16-21}

Experimental studies have confirmed that stress increases the severity and progression of periodontal disease, and several agents have been advocated to prevent stress-induced periodontitis.²²⁻²⁴ However, data on the efficacy of CAPE in periodontitis with

stressed animals are lacking. We hypothesized that administering CAPE on stressed rats may reduce alveolar bone loss and oxidative stress in periodontitis. Hence, the present study was designed to evaluate the effect of CAPE on experimental periodontitis with stressed rats.

MATERIALS AND METHODS

Animals

Forty male Sprague Dawley rats (12 weeks of age) weighing approximately 250 g were used in the present study. All rats were housed five per cage at the ideal room temperature of 21°C ± 2°C with 12-h light and dark cycle and were fed rat pellets and water ad libitum. The rats were assigned randomly to four groups as follows: control, rats with experimental periodontitis (EP), rats with EP and CS (EP-CS), and rats with EP-CS treated by CAPE (EP-CS-CAPE). The experimental protocols were approved by Pamukkale University Ethics Committee for Animal Experimentation (PAUHADYEK-2017/07). Study procedures were applied at the Pamukkale University Laboratory of Experimental Animals Research Centre.

Induction of Experimental Periodontitis

Periodontitis was induced in rats by an lipopolysaccharide (LPS) procedure (10 µL *Esherichia coli* E, serotype 055: B5, L2637; Sigma Chemical Co., St. Louis, MO, USA; 1mg/mL).²⁵⁻²⁷ General anesthesia was performed with mixture solutions of xylazine (10 mg/kg) and ketamine (40 mg/kg). Under general anesthesia, an LPS solution was administered into the vestibular gingival sites between the upper right first and second maxillary molars. This procedure was applied every other day for five days. Pure saline was administered in the control animals using the same procedure.

Protocol of Chronic Stress

Restraint stress was applied according to a previous study²⁴, using a flexible plastic mesh (30×30 cm), after which the rats were placed in plastic pipes (10 ×30 cm) for 12 hours. These animals did not receive food or water during this period. Other groups were fed limited food and water (pair feeding), but the restraint procedure was not applied. All animals were returned to their cages at

the end of the day. These protocols were performed daily for 15 days before LPS injection and 14 days after LPS injection.

Antioxidant Treatment

After the LPS injection, CAPE was administered intraperitoneally at a daily rate of 10 mmol/kg in the EP-CS-CAPE group during the last 14 days as previously described.^{19, 21, 28} The solution of CAPE was injected at the same time every day for standardization during the trials. Pure saline solutions were administered to the control, EP, and EP-CS groups using the same method.

Sample Collection

After the rats had been anesthetized intramuscularly, blood samples from the heart were collected via cardiac puncture, and the rats were decapitated. The blood samples were centrifuged at 2358 g for 10 min at 4°C to obtain serum. The serum samples were frozen at -80°C for biochemical analyses. Additionally, the maxillae of all rats were removed and fixed in 10% neutral formaldehyde solution for histological evaluation to detect alveolar bone loss.

Biochemical Assay

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

Commercially available ELISA kits (Rel Assay Diagnostics, Gaziantep, Turkey) were used to evaluate the serum total antioxidant status (TAS) and serum total oxidant status (TOS) levels according to the manufacturer's recommended protocols. The results were stated as millimolar Trolox equivalent per liter (mmol Trolox Eq/L protein) for TAS. The results were also stated as micromolar hydrogen peroxide equivalent per liter (mmol H₂O₂ Eq/L protein) for TOS. In the present study, the oxidative stress index (OSI) was calculated as described in a previous study and expressed as the percentage ratio of TOS to TAS.²⁹

Histologic Procedures

The fixed jaws (10% neutral buffered formaldehyde) were decalcified with 6% nitric acid solution for 7 days. These maxillary tissues were

stored at room temperature, and the nitric acid solution was replaced daily. At the end of this process, the decalcified tissues were kept in alcohol for dehydration and were embedded in paraffin buccolingually. Eight slides (5-mm thicknesses) were obtained for each rat by a microtome (Leica RM2125RT, Leica Instruments, Nubloch, Germany). The sections were stained using Crossman-modified Mallory triple and photographed by a light microscope with a camera attachment (Nikon Eclipse i50; Nikon, Tokyo, Japan). In the present study, the attachment level of the maxillary molars was determined by stereological analyses. The proportions of distal periodontal bone support (DPBS) and mesial periodontal bone support (MPBS) (%) were evaluated using a triocular light microscope attached to analyzing software (Kameram SLR, 1.4.1.0, Mikro Sistem, Istanbul, Turkey). To analyze the bone support, the distance between the epithelial attachment and root apex was divided by the distance between the crown tip and root apex.

Immunohistochemical Staining

For immunohistochemical analyses, the tissue sections were stained using with anti-RANKL kit (1:50 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA.) according to the manufacturer's protocols. The binding of antibodies was imaged with a high-power light microscope (Eclipse i50, Nikon, Tokyo, Japan.) to evaluate the immunopositive cell intensity. The stereologic optical fractionator method was applied to determine the numeric density values of RANKL- positive cells in 10 sections of alveolar bone for each animal. A stereology workstation, which consisted of stereology software (Stereo- Investigator, v.9.0, Microbrightfield, Williston, VT.) and a modified light microscope (Leica DM4000B, Leica Instruments.), was used to perform the stereologic analyses.³⁰

Statistical Analysis

All data had a normal distribution and the coefficient variation was less than 20%. For this reason, one-way ANOVA and the Duncan post hoc test were used with statistical software (SPSS v.17.0, IBM, Chicago, IL.) to estimate the differences among the groups. The results are

expressed as mean ± SD for each group. $p < 0.05$ was considered significant.

RESULTS

The biochemical results for each group are shown in Table 1. The serum OSI level increased

significantly in the EP group compared with the control group ($p < 0.05$). However, the CAPE treatment significantly reduced the OSI level in the EP-CS-CAPE group compared with that of the EP-CS group ($p < 0.05$).

Table 1. Comparison of Immunohistochemical and Biochemical Results

	MPBS (%)	DPBS (%)	IL-1 β	OSI	RANKL
Control	59.63±6.18*	58.27±5.95*	60.12±7.13*	2.13±0.88*	0.0000431*
EP	48.79±4.51**	49.33±4.92**	89.94±8.35**	9.39±2.40**	0.0000619**
EP-CS	39.41±3.76#	38.17±3.62#	115.52±12.82#	21.59±5.61#	0.0000825#
EP-CS-CAPE	46.94±4.38**	47.05±4.49**	94.71±10.42**	15.86±4.72##	0.0000648**

Footnote: Values are expressed as mean±SD. Symbols (*, **, #, ##) in the same column indicate significant differences among groups; $p < 0.05$.

The serum IL-1 β level in the EP group was significantly higher than that in the control group ($p < 0.05$). Furthermore, CAPE administration reduced the serum IL-1 β level in the EP-CS-CAPE group significantly more than in the EP-CS group ($p < 0.05$).

Immunohistochemical results

The level of the RANKL-positive osteoclastic cells was determined to be significantly higher in the test groups compared with the control group ($p < 0.05$). The increase of the RANKL-positive osteoclastic cells was more significant in the EP-CS group than in the EP group ($p < 0.05$). CAPE treatment significantly reduced the level of RANKL-positive osteoclastic cells in the EP-CS-CAPE group compared with the EP-CS ($p < 0.05$) (Table 1).

Bone Support

MPBS and DPBS was significantly lower in the disease groups compared with the control group ($p < 0.05$). MPBS and DPBS were significantly higher in the EP group compared with the EP-CS group ($p < 0.05$). In addition, CAPE treatment significantly decreased the loss of MPBS and DPBS in the EP-CS-CAPE group compared with that in the EP-CS group ($p < 0.05$) (Table 1).

DISCUSSION

Stress is one of the factors leading to periodontitis and the effects of stress on periodontal destruction have been established.²⁴ In this study, alveolar bone loss was significantly higher in the EP-CS group than in the EP group, confirming that stress

increases the severity of periodontitis.^{24, 31} Different agents have been used to prevent alveolar bone loss caused by stress-induced periodontitis.^{22, 23} However, to the best of our knowledge, this is the first study to evaluate the positive effects of CAPE on CS-induced periodontitis. Kazancioğlu *et al.*¹⁹ showed that CAPE significantly increased bone healing in calvarial defects. In another study, CAPE was reported to decrease alveolar bone loss significantly in experimental periodontitis.³² The present findings showed that CAPE significantly prevented alveolar bone loss in the EP-CS-CAPE group compared with the EP-CS group, and these results confirmed that CAPE reduces bone loss and enhances bone healing.^{19, 32}

RANKL controls bone formation by osteoclast cells activity and is associated with periodontal bone loss.³³ In the present study, RANKL levels were significantly higher in the EP group than in the control group, confirming that periodontitis increases the RANKL level.^{30, 33} In addition, stress can stimulate a rise in the RANKL level. Wang *et al.*³⁴ reported that stress causes myocardial injury by improving the RANKL level. Additionally, Peruzzo *et al.*²⁴ reported that CS elevates the RANKL level in periodontitis. Our results revealed that CS significantly increased the RANKL level, and these findings confirmed those of the previous studies.^{24, 34} CAPE prevents osteoclastogenesis by decreasing the RANKL level.¹⁷ The results showed that the RANKL level was significantly lower in the EP-CS-CAPE group than in the EP-CS group, and the present results in this study confirmed that CAPE

may improve bone healing by reducing RANKL-induced osteoclast activity.¹⁷

Oxidative stress causes the destruction of periodontal tissues. OSI is defined by TOS/TAS status, and we used OSI to evaluate oxidative stress. A study reported that periodontitis significantly altered OSI levels by elevating the TOS level and reducing the TAS level.³⁵ Similar to the previous study, the present results indicated that OSI was significantly higher in the EP group than in the control group. The CS can increase the OSI level by disturbing the balance of oxidant and antioxidant activity. Samarghandian *et al.*³⁶ determined that CS increased oxidative markers and decreased antioxidant markers. Our findings indicated that CS significantly increased the OSI level in the EP-CS group compared with the EP group. The present results confirmed that CS increases the ROS level and oxidative markers.^{36,37} Antioxidant agents have been used to inhibit oxidative damage. A study reported that CAPE treatment significantly reduced oxidative stress in rats with periodontitis.³² In the present study, these findings show that CAPE treatment significantly decreased the OSI level in the EP-CS-CAPE group compared with the EP-CS group and these results are consistent with those of the previous study.³² The decrease in the OSI level may be related to CAPE preventing the activation of the HPA axis and decreasing the brain corticosterone levels.

Proinflammatory cytokines have a critical role in the inflammatory process and on bone loss. Serum IL-1 β levels have been used to evaluate the severity of periodontitis. Agarwal *et al.*³⁸ reported that LPS induces the secretion of IL-1 β . Kose *et al.*³⁹ indicated that periodontitis significantly elevated the serum IL-1 β level in rats. The present findings showed that the serum IL-1 β level was significantly higher in the EP group compared with the control group, and these findings are consistent with those of previous studies.^{38,39} Psychological factors can change the production and secretion of proinflammatory cytokines, especially IL-1. Gomes *et al.*²³ suggested that stress significantly increases the hippocampal IL-1 β level in the brain; however, in the periodontium, they did not determine significantly different IL-1 β levels in the

stress-induced group compared with the control group. In another study, Peruzzo *et al.*²⁴ suggested that CS significantly elevated proinflammatory cytokines in rats with EP. Data from the present study showed that CS significantly increased serum IL-1 β levels in the EP-CS group compared with the EP group, and these results confirmed those of the previous study.²⁴ In addition, CAPE treatment significantly decreased serum IL-1 β levels in the EP-CS-CAPE group compared with the EP-CS group. CAPE has an anti-inflammatory effect and may modulate the expression of CS-induced proinflammatory cytokines.

CONCLUSIONS

Immunohistochemical and biochemical analyses showed that CS accelerates alveolar bone loss in periodontitis by stimulating the inflammatory response and oxidative stress. However, CAPE significantly prevented alveolar bone loss by reducing CS-induced RANKL, oxidative stress, and proinflammatory cytokines. The present study provided a new insight in terms of understanding effect of CS on periodontal destruction. Also, CAPE may supply antioxidant and anti-inflammatory agents to prevent the destructive effect of CS on periodontitis. However, additional studies are necessary to verify the present data and to investigate the role of other pathways in periodontitis after CS.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Deneysel Periodontitis Oluşturulan Kronik Stresli Sıçanlarda Kafeik Asit Fenetil Ester (KAPE) Uygulamasının Değerlendirilmesi

ÖZ

Amaç: Bu çalışmanın amacı sistemik kafeik asit fenetil ester (KAPE) uygulamasının lipopolisakkarit (LPS) ile periodontitis oluşturulan kronik stresli sıçanlarda oksidatif stres ve alveolar kemik kaybı üzerine terapötik etkisinin değerlendirilmesidir. **Gereç ve Yöntemler:** Çalışmamızda 40 adet Sprague Dawley sıçan kullanıldı ve bu sıçanlar dört gruba ayrıldı: 1) kontrol grubu, 2)

deneySEL periodontitis (DP) grubu, 3) DP ve kronik stres (KS) grubu (DP-KS) ve 4) KAFE ile tedavi edilen DP-KS grup (DP-KS-KAFE). Periodontitis oluşturmak amacıyla LPS test gruplarında bukkal dişetine uygulandı ve aynı yöntemle kontrol grubuna salın verildi. Bununla birlikte test gruplarından ikisine kısıtlama stresi uygulandı ve bu gruplardan biri de günlük tek doz KAFE ile tedavi edildi (10 mmol/kg). Benzer şekilde kontrol ve interlökin (IL)-1 β seviyeleri incelendi. DP ve DP-KS gruplarına da salın uygulandı. 14 gün sonra, hayvanların kalbinden serum örnekleri toplandı ve bütün hayvanlar analiz için sakrifiye edildi. Oksidatif stres, interlökin (IL)-1 β incelendi. Reseptör aktivatör nükleer faktör kappa B ligand (RANKL) ve alveolar kemik kaybı immünohistokimyasal olarak değerlendirildi. **Bulgular:** Oksidatif stres, alveolar kemik kaybı, IL-1 β ve RANKL seviyeleri DP-KS grubunda kontrol ve DP gruplarına göre anlamlı bir şekilde yüksek bulundu ($p < 0,05$). Ancak, KAFE uygulaması oksidatif stres ve IL-1 β seviyelerini DP-KS-KAFE grubunda DP-KS grubuna göre anlamlı olarak azalttı ($p < 0,05$). Bununla birlikte yine KAFE tedavisi RANKL ve alveolar kemik kaybını DP-KS-KAFE grubunda DP-KS grubuna göre anlamlı olarak azalttı ($p < 0,05$). **Sonuçlar:** Bu sonuçlar KAFE'nin immün cevabı ve enflamatuvar olayları düzenleyerek alveolar kemik kaybını önleyebileceğini göstermiştir. **Anahtar Kelimeler:** Alveolar kemik kaybı, antioksidanlar, periodontitis

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