Int. J. Plant Bas. Pharm. (2022) 2(1), 42-50

INTERNATIONAL JOURNAL OF PLANT BASED PHARMACEUTICALS

https://ijpbp.com

RESEARCH ARTICLE

OPEN ACCESS

Effects of systemic hydroxytyrosol application in experimental periodontitis of rats

Mehmet Cihan Sengun, Sadiye Gunpinar*

Bolu Abant Izzet Baysal University, Faculty of Dentistry, Department of Periodontology, Bolu, Turkey

ARTICLE INFO

Article History:

Received: 09 December 2021
Revised: 30 December 2021
Accepted: 30 December 2021
Available online: 31 December 2021

Edited by: B. Tepe

Keywords:
Alveolar bone loss
Antioxidant
Experimental periodontitis
Hydroxytyrosol
Oxidative stress

ABSTRACT

This study aimed to determine the effects of systemically administered hydroxytyrosol (HT) on alveolar bone resorption and oxidative stress parameters in experimental periodontitis (EP). Thirty-two rats were divided randomly into four groups; 1) periodontally healthy + serum physiologic (PH-SP), 2) PH + hydroxytyrosol (PH-HT), 3) experimental periodontitis + SP (EP-SP), and 4) EP-HT. Following induction of EP, 10 mg/kg of systemic HT (test)/SP (control) was administered and continued for 14 days. The animals were euthanized on the 15th day, and the jaws were removed for histopathologic, histomorphometric, and immunohistochemical analyses. Enzyme-linked immunosorbent assay (ELISA) was used to analyze serum and gingival tissue malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) levels. The receptor activator of NF-kB ligand (RANKL), osteoprotegerin (OPG) levels, and RANKL/OPG ratio were analyzed via immunolabeling. Serum and tissue MDA, SOD, and GSH-Px levels did not differ between the groups. The immunohistochemical evaluation showed that RANKL levels and RANKL/OPG ratio in HT applied groups were significantly lower than SP applied groups. Within the limits of this study, daily administration of hydroxytyrosol at a dose of 10 mg/kg for 14 days could prevent alveolar bone destruction in experimental periodontitis. Besides, the antioxidant effect of HT could not be revealed.

1. Introduction

Periodontal disease, an inflammatory disease of the periodontium, is one of the most important causes of tooth loss. It has been characterized by the imbalance between the biofilm attached to the tooth surface and the host defense mechanism (Berezow and Darveau, 2011). With the development of periodontal disease, periodontium loses its health and bleeding on probing, periodontal pocket formation, the recession of the gingiva, loss of alveolar bone, and periodontal attachment occurs (Hornef et al., 2002; Kassebaum et al., 2014; Kinane, 2001; Schroeder and Listgarten, 1997).

The interaction between the receptor activator of nuclear factor-kappa B (RANK)-the RANK ligand (RANKL)-osteoprotegerin (OPG) plays a critical role in the alveolar bone loss associated with periodontal disease. RANKL provides activation and maturation of osteoclasts. Besides, OPG hampers RANKL to bind to the RANK receptor and inhibits osteoclastogenesis by blocking the differentiation and activation of osteoclasts. While RANKL is a potent stimulator of bone resorption, OPG acts as a bone protective molecule (Bartold et al., 2010).

Free radicals are atoms or molecules with one or more unpaired electrons in their orbits. They are readily degradable, have a short turnover time, and are quite reactive. There is a protective antioxidant system against those free radicals and their harmful effects on the human body. This antioxidant system prevents radical formation, repairs oxidative damage, cleans the damaged molecules, and prevents the organism from mutations (Young and Woodside, 2001). Under normal physiological conditions, there is a balance between the antioxidant system and free radical formation. If this equilibrium is disturbed towards the oxidative side, it causes oxidative stress and leads to tissue damage (Chapple and Matthews, 2007). Conversely, the shift to the antioxidant side will reduce the soft and hard tissue injuries, and thereby the oxidative damage can be able to reduce.

* Corresponding author:
E-mail address: coskunersadiye@gmail.com (S. Gunpinar)
e-ISSN: 2791-7509
doi: https://doi.org/10.62313/ijpbp.2022.15

Please cite this article as: Sengun, M.C., Gunpinar, S., 2022. Effects of systemic hydroxytyrosol application in experimental periodontitis of rats. International Journal of Plant Based Pharmaceuticals, 2(1), 42-50, https://doi.org/10.62313/ijpbp.2022.15.

Several animal and clinical trials have been focused on the preventive effects of antioxidant chemicals such as resveratrol (Tamaki et al., 2014), flavonoids (Formica and Regelson, 1995), and melatonin (Kose et al., 2016) to reduce the tissue damage of periodontal diseases. Additionally, it has been reported that the administration of antioxidants in the treatment of periodontal disease regulates proinflammatory cytokines and can improve tissue repair by reducing soft and hard tissue loss (Chapple and Matthews, 2007).

Hydroxytyrosol (HT) is an antioxidant found abundantly in olive and olive oil, which are the main ingredients of the Mediterranean diet. It is a phenolic compound that neutralizes reactive oxygen species via chain breaking. Phenol compounds can prevent or delay the oxidation of organic molecules. They reflect their effect via transferring a proton of their Hydrogen atom to the ROO- chain of the radical (Foti, 2007). It shows the anti-inflammatory effects by inhibiting the cyclooxygenase and lipoxygenase enzymes and inhibiting the enzyme activities of immune cells such as neutrophils, mast cells, and macrophages (Brezani et al., 2017). It has been shown that HT can be beneficial and protective against many acute and chronic diseases with its anti-inflammatory and antioxidant effects. It enhances cardiovascular health (Khurana et al., 2013), reduces the risk of endothelial dysfunction (Fabiani et al., 2008), inhibits tumor cell proliferation and DNA damage, decreases edema and tissue destruction in rheumatoid arthritis (Silva et al., 2015), and has a neuroprotective role in diabetic rats (Reyes et al., 2017).

The soft and hard tissue destruction due to oxidative stress is balanced with the antioxidant system in periodontal tissues. For this reason, the homeostatic equilibrium between reactive oxygen species and the antioxidant system is critical in periodontal destruction. HT, which is the main phenolic component of olive oil and an effective antioxidant, has been reported to have a protective role in the development of chronic inflammatory diseases. However, Zhang et al. (2021) revealed that HT suppressed osteoclast differentiation and mitochondrial stress in *in vitro* model, the role of HT in the development of periodontal disease, which is also a chronic inflammatory disease, has not been elucidated yet. In this study, we hypothesized that HT treatment could regulate oxidative stress and decrease alveolar bone resorption and inflammation during the development of periodontal disease. In light of all this information, this study aimed to investigate the histopathological, histomorphometric, and immunohistochemical effects of systemic HT administration on alveolar bone resorption. The second objective was to analyze the gingival and serum levels of malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) enzymes in experimental periodontitis (EP) model in rats.

2. Materials and methods

2.1. Animals and experimental model

This study protocol was approved by the Bolu Abant Izzet Baysal University Faculty of Medicine Ethics Committee (Decision no. 2016/41). The rats used in this study were obtained from the Bolu Abant Izzet Baysal University Experimental Animal Laboratories. 32 male Albino Wistar rats weighing 200-300 grams and 10-12 weeks of age were used. All animals were housed at Bolu Abant Izzet Baysal University Experimental Animal Application and Research Center, kept 12 hours light/dark cycle and appropriate environment temperature (20 °C ± 4 °C) with 60 to 70% humidity. They were hosted in the plastic-based wire cages and could reach food and water ad libutum.

Thirty-two rats were divided randomly into four groups:
- periodontally healthy + serum physiologic (PH-SP)
- periodontally healthy + hydroxytyrosol (PH-HT)
- experimental periodontitis + serum physiologic (EP-SP)
- experimental periodontitis + hydroxytyrosol (EP-HT)

2.2. Induction of experimental periodontitis

To induce the experimental periodontitis model, rats were anesthetized with 90mg/kg ketamine hydrochloride and 10mg/kg xylazine hydrochloride intramuscularly. The EP model was induced for each rat by placing 3/0 silk sutures around the cervix of the right and left upper second molars with some subgingival location. Sutures were kept in the area for 14 days and checked without anesthesia application.

Oral gavage applications were initiated at the next day following placing the ligatures (Silva et al., 2015). Test solution was prepared as follows: HT (purity > 85%) solution [LGC, Cas No:10597-60-1, hydroxytyrosol (purity > 85%], Toronto Ontario, Canada) was dissolved in 2% methanol and was diluted with serum physiologic (SP). Rats in the test groups received 10 mg/kg HT via gavage for 14 days, once a day in the morning. For the control groups, to contain similar methanol levels, 70 ml of SP was mixed with 1.4 ml of 2% methanol and applied with the same protocol as the test group. Rats were sacrificed on the 15th day of suturing via taking whole cardiac blood.

2.3. Histopathologic and histometric analyses

Following the sacrifice, upper jaws were collected and divided into two parts with carbon disk, and half-jaws were randomly selected for histological evaluation. Those samples were fixed in 10% neutral formaldehyde and decalcified with 10% EDTA (pH 7.4) solution. After decalcification, the tissues were washed with distilled water, passed through the increasing alcohol solutions and xylene, and embedded in paraffin. Sections with a thickness of 5 µm were taken with a microtome. Histopathologic examination was performed by staining the sections with hematoxylin & eosin.

Alveolar bone loss (ABL); was determined histomorphometrically by measuring the distance between the cement-enamel junction (CEJ) and the alveolar bone crest (AC) with a light microscope with 40X magnification. Measurements were also performed separately on mesial, furcation, and distal areas in the buccal region of the second molar by the researcher who was blinded for the study groups. The hematoxylin & eosin-stained sections were evaluated under a light microscope (Nikon Eclipse i5, Nikon Instruments Inc, Tokyo, Japan). The measurements were captured and evaluated with 'Image J' (Image J v.1.5, National Institutes of Health, Maryland, USA) program.

2.4. Immunohistochemical analyses

After deparaffinization and rehydration, tissue sections were incubated with 3% hydrogen peroxide to block endogenous enzyme activity. After washing with tap water and distilled water, antigens were recovered via incubating the section in a microwave at 200 W for 20 min with citrate buffer. After washing the samples with PBS, the samples were blocked with 2% bovine serum albumin for 20 min. After blocking, the samples were incubated overnight at + 4 °C with the RANKL (RANKL/TNFSF11 Antibody, NB100-56512, Novus Biological, Littleton, USA) and OPG (Osteoprotegerin/TNFRSF11B Antibody, NB100-56505, Novus Biological, Littleton, USA) primary antibodies. After that, the secondary antibody was ligated using a

detection kit (Osteoprotegerin/TNFRSF11B Antibody, NB100-56505, Novus Biological, Littleton, USA). After washing with PBS, samples were incubated with streptavidin-peroxidase for 10 minutes and stained with 3,3'-diaminobenzidine (DAB) chromogen. Thus, the primary antibody became visible. After immunohistochemical staining, sections were examined with a camera attached (Nikon DS-Fi1c, Nikon Instruments Inc, Tokyo, Japan), light microscope (Nikon Eclipse i5, Nikon Instruments Inc, Tokyo, Japan), and evaluated with an image analysis system (NIS Elements v. 4.0, Nikon Instruments Inc, Tokyo, Japan). Intensities of RANKL and OPG staining and staining areas were measured in all sections. The staining intensity was determined as the intensity of the pixel in the computer image taken from the sections. The staining area was calculated with the ratio of the stained cell area (µm) to the entire stained area [range of interest (µm)]. Measurements were made at three regions randomly selected at 200X magnification in the whole section.

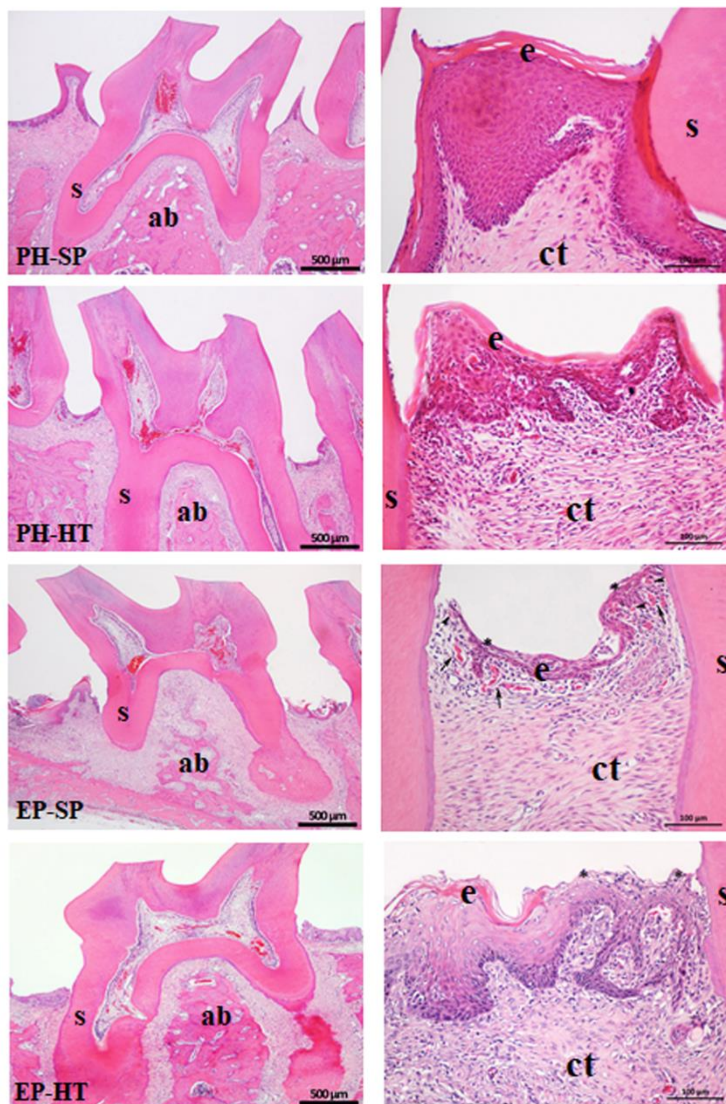

Figure 1. Alveolar bone loss and histopathological images of the study groups

Normal morphology epithelium and connective tissue in PH-SP and PH-HT groups. Spillage (*), vascularization (→), and mild, severe inflammation (›) in the keratin layer and surface epithelial cells of the EP-SP group. Spillage (*) in the keratin layer and surface epithelium in the EP-HT group. PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, e: epithelium, ct: connective tissue, s: cementum. Hematoxylin & eosin staining, 40X magnification, bar: 500 µm (left images) 200X magnification, bar: 100 µm (right images).

2.5. Biochemical analyses

The gingival tissue samples with a total size of 3 mm were obtained around the sutures and immediately stored at -80 °C in sterile tubes containing 400 µl phosphate-buffered saline (PBS) (Panbiotech, P04-31500, 500ml, Germany) and 0.05% Tween-20 until analysis. On the day of analysis, samples were dissolved in PBS with the ratio of 100mg tissue/100µl PBS, transferred to tubes, and each sample was disintegrated entirely with tissue homogenizer (IKA WERKE, Wilmington, USA) at least 10 minutes. Freeze-thawing was applied to homogenates which were homogenized in 1 ml PBS, twice at -20 °C, and following the final thawing, samples were centrifuged, and the supernatants were separated for analysis.

To separate the serum component of cardiac blood, samples were centrifuged (1200 g, 10 minutes) at +4 °C, and aliquoted serum samples were stored at -80 °C until assayed.

2.6. Serum and gingival tissue levels of MDA, SOD, and GSH-Px

Malondialdehyde (MDA) (Cayman Rat MDA, Cat. No: 706002, Cayman Chemical Inc. Michigan, USA), glutathione peroxidase (GSH-

Px) (Cayman Rat Gsh-Px, Cat. No: 10009055, Cayman Chemical Inc. Michigan, USA) and superoxide dismutase (SOD) (Cayman Rat SOD, Cat. No: 703102, Cayman Chemical Inc. Michigan, USA) levels in the serum and gingival tissue samples were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. The linear measurement range was 0-50 nmol/ml, 50-344 nmol/min/ml, and 0.005-0.05 units/ml for MDA, GSH-PX and SOD, respectively. The samples out of the linear range were reevaluated with appropriate dilutions.

In order to assess toxicity, gamma-glutamyl transferase (GGT) (U/L), aspartate transaminase (AST) (U/L), alanine aminotransferase (ALT) (U/L), alkaline phosphatase (ALP) (U/L), blood urea nitrogen (UREA) (mg/dL) and creatinine (CREA) (mg/dl) levels were evaluated. Measurements were done spectrophotometrically using enzymatic and colorimetric methods using the manufacturer's instructions (Architect c 8000, Abbott Laboratuvari, Illinois, USA).

2.7. Statistical analyses

For all evaluated parameters, the normality was tested by the Shapiro-Wilk test. If the data distribution was normal, a parametric test, one-way ANOVA-Tukey, otherwise a nonparametric test, Kruskal Wallis-Mann-Whitney U test was employed to determine the differences between groups. All analyses were performed using the SPSS 19 (Statistical Package for Social Sciences) package program, and values of $p < 0.05$ were considered significant.

3. Results and discussion

3.1. Histopathologic and histomorphometric findings

Images of histological sections are given in Figure 1. Levels of bone loss in the study group were summarized in Table 1. Measurements of alveolar bone loss in EP groups were statistically higher than those of PH groups ($p < 0.05$). On the other hand, there was no statistically significant difference between experimental periodontitis groups (EP-SP and EP-HT) ($p > 0.05$).

Table 1. Alveolar bone loss measurement values in the study groups (µm)

	PH-SP (n=8)	PH-HT (n=8)	EP-SP (n=8)	EP-HT (n=8)	$p^{\alpha,\beta}$
Mesial					
Mean ± sd	195.63 ± 50.40	154.16 ± 21.34	595.23 ± 193.66^{a,b}	437.68 ± 203.49^{a,b}	0.000$^{\alpha}$*
Median	191.79	160.37	563.22	371.19	
Min-Max	122.52-302.92	120.02-181.24	358.97-894.36	249.89-767.41	
Furcation area					
Mean ± sd	117.78 ± 23.37	98.35 ± 12.30	406.99 ± 183.97^{a,b}	318.66 ± 211.54^{a,b}	0.000$^{\beta}$*
Median	113.45	96.98	394.05	228.83	
Min-Max	91.73-152.06	80.86-118.75	181.14-639.24	151.19-775.31	
Distal					
Mean ± sd	516.01 ± 132.74	519.48 ± 154.27	1124.76 ± 352.79^{a,b}	856.59 ± 293.52^{a}	0.000$^{\alpha}$*
Median	486.00	491.71	1036.42	854.84	
Min-Max	358.62-762.59	288.30-754.36	755.02-1858.73	524.98-1312.39	
Total					
Mean ± sd	276.47 ± 65.05	257.33 ± 55.45	708.99 ± 194.52^{a,b}	537.64 ± 198.86^{a,b}	0.000$^{\alpha}$*
Median	270.57	242.70	678.96	497.53	
Min-Max	193-48-405.86	183.08-351.45	464.10-1086.02	324.87-868.62	

$^{\alpha}$One Way ANOVA, $^{\beta}$Kruskal Wallis. *$p < 0.05$. n: number of samples.
a: Difference from PH-SP, b: Difference from PH-HT.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, µm: Micrometer, sd: Standart deviation, Min: Minimum, Max: Maximum.

3.2. RANKL, OPG, and RANKL/OPG immunohistochemical findings

RANKL staining of 2 of the 32 hard tissue samples could not be evaluated because of the pouring and folding of the sections. Both the staining area and intensity levels of RANKL and OPG were summarized in Table 2, and the images of the sections were depicted in Figure 2.

The RANKL staining area in the EP-HT group was statistically lower than that of the EP-SP group ($p < 0.05$). When the levels of OPG staining area were compared between groups, it was found that these levels were low in the EP-SP group than PH-SP group as expected, but the results did not reach significance ($p > 0.05$). The RANKL/OPG staining area ratio was significantly higher in the PH-SP group than in the PH-HT and EP-HT groups ($p < 0.05$).

The RANKL staining intensity of the EP-HT group was significantly lower than the PH-SP group ($p < 0.05$). It was determined that EP-SP group RANKL staining intensity was significantly higher than that of PH-HT ($p < 0.05$); on the other hand, these measurements were lower in the EP-HT than EP-SP group ($p < 0.05$). When the OPG staining intensity was compared, then the levels of PH-SP and PH-HT groups were significantly higher than those of EP-SP and EP-HT groups ($p < 0.05$). The RANKL/OPG staining intensity of the EP-SP group was found to be higher than the other groups ($p < 0.05$).

3.3. Biochemical analyses

3.3.1. Serum MDA, SOD and GSH-Px levels

Serum MDA, SOD, and GSH-Px levels in both groups were summarized in Table 3. Measurements of the level of these enzymes did not differ between the groups ($p > 0.05$).

3.3.2. Gingival tissue MDA, SOD, and GSH-Px levels

The tissue MDA levels of the EP groups were higher than those of PH control, but the results were not statistically significant ($p > 0.05$). When the tissue levels of SOD were compared between groups, it was determined that the levels in the EP-induced groups were lower than the PH-HT group ($p < 0.05$). On the other hand, the levels of SOD were found to be increased in the EP group with HT treatment, but the results did not reach significance ($p > 0.05$) (Table 3).

3.3.3. Serum GGT, AST, ALT, ALP, UREA, and CREA

It was determined that the enzyme mentioned above levels were below the toxic level, and these levels were not statistically significant between the groups ($p > 0.05$) (Table 4). Serum GGT levels, a marker of liver toxicity, were under the toxic levels in all groups (< 4 U/L); therefore, these results were not given in the tables.

This study evaluated the effects of 10 mg/kg systemic HT administration on alveolar bone destruction and oxidative stress parameters in rats with EP. We revealed that systemic HT administration decreased the level of RANKL staining. On the other hand, although the decreased alveolar bone loss was determined in HT groups via morphometric analysis, the results did not reach significance. Besides, tissue and serum MDA, SOD, and GSH-Px levels did not differ between those treated with HT or SP in periodontitis groups. Therefore, the results of this study showed that 10 mg/kg systemic HT administration could not reduce the reactive oxygen species and could not present antioxidant properties.

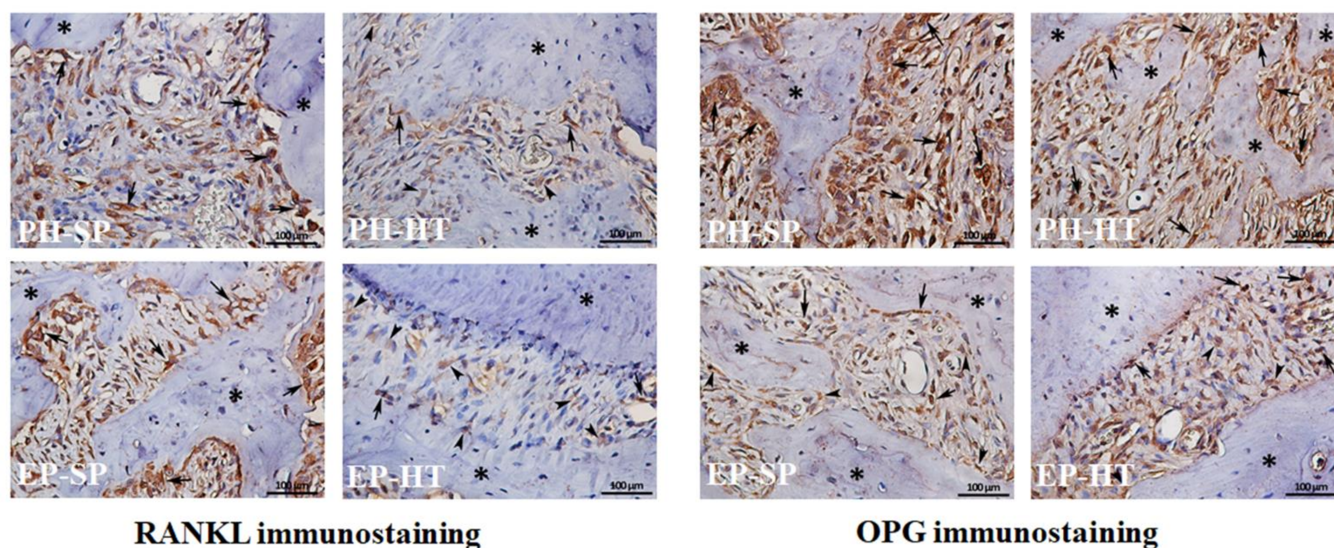

Figure 2. Histological images of RANKL and OPG immunostaining in the experimental groups

(→): Severe immune-positive stained cells, (›): Weakly immune-positive stained cells., (*): Alveolar bone. PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol. (200X magnification, bar: 100 µm.)

Table 2. RANKL and OPG immunohistochemical results

	PH-SP (n=7)	PH-HT (n=7)	EP-SP (n=8)	EP-HT (n=8)	$p^{\alpha,\beta}$
Staining area					
RANKL (+) cell number (%)					
Mean ± sd	5.79 ± 1.15	4.24 ± 1.02	7.76 ± 2.94 ^{b,d}	2.65 ± 1.11	0.000$^{\alpha}$ *
Median	6.11	3.95	6.82	2.95	
Min-Max	4,11-7,65	3.19-6.38	3.68-13.15	0.93-4.04	
OPG (+) cell number (%)					
Mean ± sd	24.99 ± 7.92	34.61 ± 9.72	25.19 ± 5.93	22.67 ± 5.97	0.084$^{\beta}$
Median	25.05	33.48	22.94	24.41	
Min-Max	10.24-34.02	25.89-54.92	18.05-34.70	19.17-33.66	
RANKL/OPG					
Mean ± sd	0.26 ± 0.16^{b,d}	0.12 ± 0.03	0.32 ± 0.14^{b,d}	0.11 ± 0.06	0.010$^{\beta}$ *
Median	0.24	0.12	0.28	0.10	
Min-Max	0.12-0.62	0.06-0.18	0.15-0.58	0.03-0.20	
Staining intensity					
RANKL					
Mean ± sd	66.80 ± 9.96^{d}	54.52 ± 9.02	77.75 ± 8.84^{b,d}	52.41 ± 12.34	0.010$^{\beta}$ *
Median	63.66	56.33	82.5	54.5	
Min-Max	54.66-82.00	36.00-62.33	60.66-85.00	32.00-68.33	
OPG					
Mean ± sd	93.04 ± 9.59^{c,d}	92.76 ± 8.68^{c,d}	65.37 ± 8.03	75.19 ± 8.48	0.000$^{\alpha}$ *
Median	92.00	91.66	66.83	73.00	
Min-Max	79.33-109.66	82.00-104.33	49.33-73.66	64.66-91.66	
RANKL/OPG					
Mean ± sd	0.73 ± 0.16	0.57 ± 0.09	1.20 ± 0.17^{a,b,d}	0.69 ± 0.13	0.000$^{\beta}$ *
Median	0.75	0.58	1.16	0.76	
Min-Max	0.54-1.03	0.42-0.71	0.96-1.48	0.45-0.82	

$^{\alpha}$One Way ANOVA, $^{\beta}$Kruskal Wallis. *$p < 0.05$. n: number of samples.
a: Difference from PH-SP, b: Difference from PH-HT, c: Difference from EP-SP, d: Difference from EP-HT.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, RANKL: The receptor activator of NF-κB ligand, OPG: Osteoprotegerin, sd: Standart deviation, Min: Minimum, Max: Maximum.

In our study, the amount of alveolar bone loss was calculated by measuring the distance between CEJ and AC in the second molars of rats. The amount of alveolar bone loss was higher in the experimental periodontitis groups compared to the PH groups. Although HT treatment in the EP group showed a decreased bone loss, the results were not statistically significant. On the other hand,

Zhang et al. (2021) determined decreased alveolar bone loss in mice periodontitis model treated with HT via Micro-CT analysis. The researchers applied 20 mg/kg HT one week before the induction of periodontitis and continued for two weeks. Conversely, in our study, 10 mg/kg HT was administered concurrently with the periodontitis induction. Therefore, our results might be due to the fact that the relatively low doses of antioxidants would not be able to prevent bone resorption. Furthermore, only two-dimensional measurement was utilized rather than calculating the bone loss volume.

Table 3. Serum and gingival tissue MDA (nmol/ml), SOD (U/ml) and GSH-Px (nmol/min/ml) levels in the study groups

	PH-SP (n=8)	PH-HT (n=8)	EP-SP (n=7)	EP-HT (n=8)	p^{Ω}
Serum levels					
MDA (nmol/ml)					
Mean ± sd	4.01 ± 0.70	4.42 ± 1.08	4.37 ± 0.69	4.54 ± 1.26	0.728
Median	4.00	4.44	4.22	4.63	
Min-Max	3.04-5.10	2.18-5.53	3.55-5.41	1.84-5.78	
SOD (U/ml)					
Mean ± sd	0.74 ± 0.13	0.55 ± 0.11	0.65 ± 0.11	0.60 ± 0.22	0.117
Median	0.76	0.58	0.66	0.61	
Min-Max	0-54-0.98	0.41-0.71	0.47-0.81	0.15-0.86	
GSH-PX (nmol/min/ml)					
Mean ± sd	147.25 ± 46.19	114.12 ± 64.22	143.50 ± 44.25	160.37 ± 60.23	0.401
Median	166.00	101.00	128.50	151.00	
Min-Max	63.50-198.50	33.50-243.50	93.50-203.50	93.50-293.50	
Gingival tissue levels					
MDA (nmol/ml)					
Mean ± sd	0.86 ± 0.45	0.99 ± 0.70	0.94 ± 0.12	0.79 ± 0.43	0.848
Median	0.70	0.80	0.93	0.79	
Min-Max	0.45-1.75	0.40-2.41	0.79-1.16	0.25-1.46	
SOD (U/ml)					
Mean ± sd	8.23 ± 2.26	10.12 ± 1.87[a,b]	7.21 ± 1.23	7.39 ± 1.89	0.015*
Median	7.23	9.46	6.94	7.41	
Min-Max	5.81-12.29	8.19-13.83	6.24-9.35	5.20-10.93	
GSH-PX (nmol/min/ml)					
Mean ± sd	1.29 ± 0.58	1.86 ± 0.51	1.20 ± 0.44	1.55 ± 0.86	0.170
Median	1.39	1.79	1.19	1.38	
Min-Max	0.35-2.19	1.23-2.22	0.80-1.96	0.42-3.07	

$^{\Omega}$One Way ANOVA, *$p < 0.05$. n: number of samples.
a: Difference from EP-SP, b: Difference from EP-HT.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, MDA: Malondialdehyde, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, nmol/ml: nanomole/milliliter, U/ml: Units per millilitre, sd: Standart deviation, Min: Minimum, Max: Maximum.

Table 4. Serum AST (U/L), ALT (U/L), ALP (U/L), UREA (mg/dl) and CREA (mg/dl) levels in the study groups

	PS-SF (n=8)	PS-HT (n=8)	DP-SF (n=7)	DP-HT (n=8)	p^{Ω}
AST (U/L)					
Mean ± sd	129.50 ± 41.57	97.50 ± 10.90	104 ± 14.92	95.87 ± 12.19	0.610
Median	109.00	96.00	103.00	93.00	
Min-Max	98.0-219.0	83.0-114.0	81.0-128.0	84.0-120.0	
ALT (U/L)					
Mean ± sd	62.37 ± 10.08	51.12 ± 6.53	57.71 ± 7.11	55.12 ± 11.95	0.445
Median	61,50	52,50	60.00	58.00	
Min-Max	48.0-78.0	41.0-59.0	48.0-67.0	29.0-67.0	
ALP (U/L)					
Mean ± sd	240.62 ± 26.04	208.75 ± 51.28	217 ± 39.77	242.75 ± 71.39	0.128
Median	238.50	206.00	201.00	258.00	
Min-Max	206.0-288.0	110.0-282.0	173.0-283.0	116.0-332.0	
UREA (mg/dl)					
Mean ± sd	50.87 ± 5.43	50.75 ± 4.30	48.42 ± 2.50	53.75 ± 5.99	0.329
Median	(43-58)	(43-58)	(45-51)	(45-60)	
Min-Max	43.0-58.0	43.0-58.0	45.0-51.0	45.0-60.0	
CREA (mg/dl)					
Mean ± sd	0.54 ± 0.02	0.51 ± 0.02	0.52 ± 0.03	0.52 ± 0.02	0.294
Median	0.54	0.51	0.52	0.52	
Min-Max	0.51-0.59	0.49-0.55	0.50-0.60	0.48-0.56	

$^{\Omega}$One Way ANOVA, *$p < 0.05$. n: number of samples.
PH-SP: Periodontally healthy + serum physiologic, PH-HT: Periodontally healthy + hydroxytyrosol, EP-SP: Experimental periodontitis + serum physiologic, EP-HT: Experimental periodontitis + hydroxytyrosol, AST: Aspartate transaminase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, UREA: Blood urea nitrogen, CREA: Creatine, U/L: Units per liter, mg/dl: Milligrams per deciliter, sd: Standart deviation, Min: Minimum, Max: Maximum.

The interaction of RANK-RANKL-OPG plays a critical role in alveolar bone destruction associated with periodontal disease as well as systemic conditions such as osteoporosis. Although the alteration in RANKL and OPG levels are utilized alone, it is more valid to determine the RANKL/OPG ratio to reveal the alveolar bone loss. In the literature, immunohistochemical evaluation of RANKL and OPG was presented by staining area and/or staining intensity. The 'intensity' of the staining indicates the degree of expression of the cells in the histological sections, while the staining area refers to the number of expressing cells within a specific area in the sections (Fedchenko and Reifenrath, 2014). The staining intensity is assessed by computer software which gives precise values, while the staining area was determined via counting the stained cells in the section separately by the researchers to record numerical values. Assessment of the staining area is a more practical technique commonly used in the literature; on the other hand, the staining

intensity offers more detailed information but requires more time (Fedchenko and Reifenrath, 2014). This study evaluated both staining area and staining intensity of RANKL and OPG levels and RANKL/OPG ratio. In line with the literature, RANKL levels and RANKL/OPG ratio were increased, and OPG levels were decreased in periodontal disease (Arabacı et al., 2015; Saglam et al., 2015). Moreover, considering the results of this study, it can be concluded that the assessment of staining intensity rather than staining area reveals more sensitive results in immunohistochemical analysis.

Previous studies have shown that alveolar bone loss can be prevented by systemic administration of various antioxidant agents as a result of decreased RANKL and increased OPG levels (Arabacı et al., 2015; Saglam et al., 2015). In this study, the effect of HT administration on RANKL and OPG levels were evaluated, and both staining area and staining intensity values of RANKL were decreased, but OPG levels did not change in EP by HT administration. This can be explained by the fact that the need for OPG, a competing inhibitor of RANKL, may be reduced as a result of decreased RANKL levels (Zhou et al., 2013). When RANKL/OPG ratio was compared, systemic HT application decreased both the staining area and the intensity levels in EP. These results were supported with the previous studies showing decreased RANKL level, RANKL/OPG ratio, and increased OPG levels after the administration of various antioxidants such as sumac (Saglam et al., 2015), resveratrol (Tamaki et al., 2014), melatonin (Arabacı et al., 2015) and curcumin (Zhou et al., 2013) in periodontal disease.

In the literature, it has been reported that HT could be applied in different amounts such as 2 mg/kg (Mnafgui et al., 2016), 5 mg/kg (Silva et al., 2015), and 10 mg/kg (Pirozzi et al., 2016); the physiological lower limit has been reported as 5 mg/kg by the European Food Safety Authority (Cristina Vilaplana-Perez et al., 2014). Pirozzi et al. (2016) reported that HT administration at a dose of 10 mg/kg per day reduces liver damage and regulates oxidative stress by demonstrating anti-inflammatory and antioxidant characteristics without showing any toxic reaction (Pirozzi et al., 2016). Furthermore, pretreatment with 20 mg/kg HT partially decreased alveolar bone loss in a mice periodontitis model (Zhang et al., 2021). In the light of this information, systemic HT administration at a dose of 10 mg/kg per day was utilized concurrently with the induction of periodontitis in this study.

Lipid peroxidation levels in biological fluids of subjects with periodontal disease are controversial. It has been reported that periodontitis patients had higher serum and tissue MDA levels than healthy controls. Besides, decreased MDA levels in GCF and saliva have reduced inflammation. On the contrary, periodontitis subjects had lower serum MDA levels than healthy individuals (Akalın et al., 2007; Baltacioglu et al., 2014; Saglam et al., 2018; Tsai et al., 2005). In our study, serum MDA levels were lower in EP than healthy controls, and tissue MDA levels were similar among groups. Furthermore, HT administration did not affect either serum or tissue MDA levels in EP. These results were compatible with the literature, which reports that administration of antioxidants did not alter the serum MDA levels (Tas et al., 2015; Wang et al., 2017).

Superoxide dismutase and GSH-Px enzyme levels in biological fluids and local tissues have been studied to clarify the antioxidant mechanism in periodontal disease. Sobaniec and Sobaniec-Lotowska (2000) reported that serum SOD, GSH-Px, and glutathione reductase enzymes were lower, and MDA levels were higher in EP-induced rats compared to the periodontally healthy group (Sobaniec and Sobaniec-Lotowska, 2000). In parallel, it was determined that the MDA levels in gingival tissue could be significantly reduced by administering the pharmacological imitation of SOD (Di Paola et al., 2005). In a clinical study, Ellis et al. (1998) found that the catalase and SOD levels in gingival tissues obtained from the areas with a periodontal pocket depth of more than 6 mm were lower than those in healthy tissues with a pocket depth of less than 3 mm (Ellis et al., 1998). In contrast, Akalin et al. (2005) analyzed SOD enzyme levels in gingival samples of 26 cases with periodontitis and 16 periodontally healthy individuals and reported that SOD levels were significantly higher in periodontitis patients than in healthy individuals (Akalin et al., 2005). Huang et al. (2000) reported that GSH-Px levels in GCF were negatively correlated with pocket depth and attachment loss in patients with chronic periodontitis, and the GCF levels were increased after periodontal treatment (Huang et al., 2000). In our study, although serum and tissue SOD and GSH-Px levels were lower in EP groups, the results did not reach significance. Additionally, HT administration did not significantly affect those except SOD levels in the periodontally healthy group. These results could be explained by the increased oxidative stress and decreased antioxidant mechanism in periodontal disease. Furthermore, antioxidant mechanisms might be activated in the later stages of chronic inflammation. These results also support the hypothesis that serum enzyme levels may not reflect the local tissue response in periodontitis (Karakan et al., 2017).

In this study, serum levels of ALT, AST, GGT, ALP, urea, and creatinine, indicative of hepatic and renal damage, were also investigated. All enzyme levels were found to be below the toxic threshold, and they were consistent with the previous studies in the literature (Dalcico et al., 2013; Franca et al., 2017; Wang et al., 2013).

The results of our study should be discussed precisely due to not having enough published papers that investigated the possible effect of HT administration on EP. In a very recently published literature, the researchers demonstrated that HT application suppressed bone destruction via the prevention of osteoclast activation *in vitro* and pretreatment with daily administration of 20 mg/kg HT partially decreased alveolar bone resorption and oxidative stress *in vivo* mice periodontitis model (Zhang et al., 2021). These researchers used the prophylactic application of HT. That might increase the tissue antioxidant levels of HT, which might prevent periodontitis-induced alveolar bone loss. Conversely, in our study, the administration of HT was started simultaneously with the induction of periodontitis. Therefore, discussing the results of that research with the current study may not be convenient.

Furthermore, the limitation of this study is that the histomorphometric measurements of alveolar bone loss were carried out in two dimensions only. The fact that a different antioxidant substance was not compared with HT and the total oxidant/antioxidant levels have not been evaluated can be considered limitations.

4. Conclusions

The hypothesis that systemically administrated HT at a dose of 10 mg/kg could reduce alveolar bone destruction was supported in immunohistochemical evaluation but not by the results of histomorphometric measurements. Therefore, it can be suggested that the effect of HT on alveolar bone loss should be further analyzed with a three-dimensional volume assessment via micro-computed tomography. Moreover, different application doses and frequencies of HT compared with different antioxidants should be investigated to clarify HT's antioxidant characteristics in EP.

Acknowledgments

This study was supported by Bolu Abant Izzet Baysal University Scientific Research Center (Project number 2017.06.05.1144). The authors would like to thank Dr. Buket Tekçe for her kind assistance in biochemical analysis.

Conflict of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Mehmet Cihan Sengun: He performed all the experimental procedures and drafted the manuscript.
Sadiye Gunpinar: She supervised all the experimental study and edited the original manuscript.

ORCID Numbers of the Authors

M.C. Sengun: 0000-0002-9293-2117
S. Gunpinar: 0000-0001-6100-322X

Supplementary File

None.

References

Akalin, F.A., Toklu, E., Renda, N., 2005. Analysis of superoxide dismutase activity levels in gingiva and gingival crevicular fluid in patients with chronic periodontitis and periodontally healthy controls. *Journal of Clinical Periodontology*, 32(3), 238-243.
Akalın, F.A., Baltacıoğlu, E., Alver, A., Karabulut, E., 2007. Lipid peroxidation levels and total oxidant status in serum, saliva and gingival crevicular fluid in patients with chronic periodontitis. *Journal of Clinical Periodontology*, 34(7), 558-565.
Arabacı, T., Kermen, E., Özkanlar, S., Köse, O., Kara, A., Kızıldağ, A., Ibişoğlu, E., 2015. Therapeutic effects of melatonin on alveolar bone resorption after experimental periodontitis in rats: a biochemical and immunohistochemical study. *Journal of Periodontology*, 86(7), 874-881.
Baltacıoğlu, E., Yuva, P., Aydın, G., Alver, A., Kahraman, C., Karabulut, E., Akalın, F.A., 2014. Lipid peroxidation levels and total oxidant/antioxidant status in serum and saliva from patients with chronic and aggressive periodontitis. Oxidative stress index: a new biomarker for periodontal disease?. *Journal of Periodontology*, 85(10), 1432-1441.
Bartold, P.M., Cantley, M.D., Haynes, D.R., 2010. Mechanisms and control of pathologic bone loss in periodontitis. *Periodontology 2000*, 53(1), 55-69.
Berezow, A.B., Darveau, R.P., 2011. Microbial shift and periodontitis. *Periodontology 2000*, 55(1), 36-47.
Brezani, V., Smejkal, K., Hosek, J., Tomasova, V., 2018. Anti-inflammatory natural prenylated phenolic compounds-potential lead substances. *Current Medicinal Chemistry*, 25(10), 1094-1159.
Chapple, I.L., Matthews, J.B., 2007. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontology 2000*, 43(1), 160-232.
Dalcico, R., de Menezes, A.M., Deocleciano, O.B., Oriá, R.B., Vale, M.L., Ribeiro, R.A., Brito, G.A.D.C., 2013. Protective mechanisms of simvastatin in experimental periodontal disease. Journal of Periodontology, 84(8), 1145-1157.
Di Paola, R., Mazzon, E., Rotondo, F., Dattola, F., Britti, D., De Majo, M., Cuzzocrea, S., 2005. Reduced development of experimental periodontitis by treatment with M40403, a superoxide dismutase mimetic. *European Journal of Pharmacology*, 516(2), 151-157.
Ellis, S.D., Tucci, M.A., Serio, F.G., Johnson, R.B., 1998. Factors for progression of periodontal diseases. *Journal of Oral Pathology & Medicine*, 27(3), 101-105.
Fabiani, R., Rosignoli, P., De Bartolomeo, A., Fuccelli, R., Servili, M., Montedoro, G.F., Morozzi, G., 2008. Oxidative DNA damage is prevented by extracts of olive oil, hydroxytyrosol, and other olive phenolic compounds in human blood mononuclear cells and HL60 cells. *The Journal of Nutrition*, 138(8), 1411-1416.
Fedchenko, N., Reifenrath, J., 2014. Different approaches for interpretation and reporting of immunohistochemistry analysis results in the bone tissue–a review. *Diagnostic Pathology*, 9(1), 1-12.
Formica, J.V., Regelson, W., 1995. Review of the biology of quercetin and related bioflavonoids. Food and Chemical Toxicology, 33(12), 1061-1080.
Foti, M.C., 2007. Antioxidant properties of phenols. *Journal of Pharmacy and Pharmacology*, 59(12), 1673-1685.
França, L.F.C., Vasconcelos, A.C.C., da Silva, F.R., Alves, E.H., Carvalho, J.S., Lenardo, D.D., Vasconcelos, D.F., 2017. Periodontitis changes renal structures by oxidative stress and lipid peroxidation. *Journal of Clinical Periodontology*, 44(6), 568-576.
Hornef, M.W., Wick, M.J., Rhen, M., Normark, S., 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. Nature Immunology, 3(11), 1033-1040.
Huang, P., Su, T., Wang, H., 2000. The relationship between GPx activity in gingival fluid and clinical parameters of adult periodontitis. *Hua xi kou qiang yi xue za zhi= Huaxi kouqiang yixue zazhi= West China Journal of Stomatology*, 18(2), 106-108.
Karakan, N.C., Akpınar, A., Göze, F., Poyraz, Ö., 2017. Investigating the effects of systemically administered strontium ranelate on alveolar bone loss histomorphometrically and histopathologically on experimental periodontitis in rats. *Journal of Periodontology*, 88(2), e24-e31.
Kassebaum, N.J., Bernabé, E., Dahiya, M., Bhandari, B., Murray, C.J.L., Marcenes, W., 2014. Global burden of severe tooth loss: a systematic review and meta-analysis. *Journal of Dental Research*, 93(7_suppl), 20S-28S.
Khurana, S., Venkataraman, K., Hollingsworth, A., Piche, M., Tai, T.C., 2013. Polyphenols: benefits to the cardiovascular system in health and in aging. *Nutrients*, 5(10), 3779-3827.
Kinane, D.F., 2001. Causation and pathogenesis of periodontal disease. *Periodontology 2000*, 25(1), 8-20.
Kose, O., Arabaci, T., Kara, A., Yemenoglu, H., Kermen, E., Kizildag, A., Ozkanlar, S., 2016. Effects of melatonin on oxidative stress index and alveolar bone loss in diabetic rats with periodontitis. *Journal of Periodontology*, 87(5), e82-e90.
Mnafgui, K., Hajji, R., Derbali, F., Khlif, I., Kraiem, F., Ellefi, H., Gharsallah, N., 2016. Protective effect of hydroxytyrosol against cardiac remodeling after isoproterenol-induced myocardial infarction in rat. *Cardiovascular Toxicology*, 16(2), 147-155.
Pirozzi, C., Lama, A., Simeoli, R., Paciello, O., Pagano, T.B., Mollica, M.P., Meli, R., 2016. Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring duodenal integrity in a rat model of NAFLD. *The Journal of Nutritional Biochemistry*, 30, 108-115.
Reyes, J.J., Villanueva, B., López-Villodres, J.A., De La Cruz, J.P., Romero, L., Rodríguez-Pérez, M.D., González-Correa, J.A., 2017. Neuroprotective effect of hydroxytyrosol in experimental diabetes mellitus. *Journal of Agricultural and Food Chemistry*, 65(22), 4378-4383.
Saglam, E., Canakci, C. F., Sebin, S. O., Saruhan, N., Ingec, M., Canakci, H., & Sezer, U. (2018). Evaluation of oxidative status in patients with chronic periodontitis and polycystic ovary syndrome: A cross-sectional study. Journal of Periodontology, 89(1), 76-84.
Sağlam, M., Köseoğlu, S., Hatipoğlu, M., Esen, H.H., Köksal, E., 2015. Effect of sumac extract on serum oxidative status, RANKL/OPG system and alveolar bone loss in experimental periodontitis in rats. *Journal of Applied Oral Science*, 23, 33-41.
Schroeder, H.E., Listgarten, M.A., 1997. The gingival tissues: the architecture of periodontal protection. *Periodontology 2000*, 13(1), 91-120.
Silva, S., Sepodes, B., Rocha, J., Direito, R., Fernandes, A., Brites, D., Figueira, M.E., 2015. Protective effects of hydroxytyrosol-supplemented refined olive oil in animal models of acute inflammation and rheumatoid arthritis. *The Journal of Nutritional Biochemistry*, 26(4), 360-368.
Sobaniec, H., Sobaniec-Lotowska, M.E., 2000. Morphological examinations of hard tissues of paradontium and evaluation of selected processes of lipid peroxidation in blood serum of rats in the course of experimental periodontitis. *Medical Science Monitor*, 6(5), 875-881.
Tamaki, N., Orihuela-Campos, R.C., Inagaki, Y., Fukui, M., Nagata, T., Ito, H.O., 2014. Resveratrol improves oxidative stress and prevents the progression of periodontitis via the activation of the Sirt1/AMPK and the Nrf2/antioxidant defense pathways in a rat periodontitis model. *Free Radical Biology and Medicine*, 75, 222-229.
Tas, U., Ayan, M., Sogut, E., Kuloglu, T., Uysal, M., Tanriverdi, H.I., Sarsilmaz, M., 2015. Protective effects of thymoquinone and melatonin on intestinal ischemia–reperfusion injury. *Saudi Journal of Gastroenterology: Official Journal of the Saudi Gastroenterology Association*, 21(5), 284-289.
Tsai, C.C., Chen, H.S., Chen, S.L., Ho, Y.P., Ho, K.Y., Wu, Y.M., Hung, C.C., 2005. Lipid peroxidation: a possible role in the induction and progression of chronic periodontitis. *Journal of Periodontal Research*, 40(5), 378-384.
Wang, H., Xue, L., Yan, R., Zhou, Y., Wang, M.S., Cheng, M.J., Huang, H.J., 2013. Comparison of histologic characteristics of Chinese chronic hepatitis B patients with persistently normal or mildly elevated ALT. *PloS One*, 8(11), e80585.
Wang, X.L., Li, T., Li, J.H., Miao, S.Y., Xiao, X.Z., 2017. The effects of resveratrol on inflammation and oxidative stress in a rat model of chronic obstructive pulmonary disease. *Molecules*, 22(9), 1529.
Young, I.S., Woodside, J.V., 2001. Antioxidants in health and disease. *Journal of Clinical Pathology*, 54(3), 176-186.
Zhang, X., Jiang, Y., Mao, J., Ren, X., Ji, Y., Mao, Y., Huang, S., 2021. Hydroxytyrosol prevents periodontitis-induced bone loss by regulating mitochondrial function and mitogen-activated protein kinase signaling of bone cells. *Free Radical Biology and Medicine*, 176, 298-311.
Zhou, T., Chen, D., Li, Q., Sun, X., Song, Y., Wang, C., 2013. Curcumin inhibits inflammatory response and bone loss during experimental periodontitis in rats. *Acta Odontologica Scandinavica*, 71(2), 349-356.

Reviewed by:

Bilge MERACI YILDIRAN: Karabuk University, Karabuk, TURKEY
Umut YIGIT: Usak University, Usak, TURKEY

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

This is an open-access article distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.