EFFECT OF PALMATINE ON PERIODONTAL TISSUE DESTRUCTION IN EXPERIMENTAL PERIODONTITIS RAT MODEL

DENEYSEL PERİODONTİTİS RAT MODELİNDE PALMATİNİN PERİODONTAL DOKU YIKIMI ÜZERİNE ETKİSİ

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Öz

Amaç: Bu çalışmanın amacı deneysel periodontitis (DP) rat modelinde oral olarak uygulanan palmatinin periodonsiyum üzerindeki etkilerini incelemektir. Gereç ve Yöntem: Toplam 40 sıçan, DP oluşturulmayan ve palmatin uygulanmayan (grup 1, CTRL); DP oluşturulan ama palmatin uygulanmayan (grup 2, EP); DP oluşturulan ve 5 mg / kg palmatin uygulanan (grup 3, EP-5P); DP oluşturulan ve 10 mg / kg palmatin uygulanan (grup 4, EP-10P) olmak üzere dört gruba ayrıldı. Deneysel periodontitis, sıçanların sağ mandibular birinci molar dişlerinin servikal kenarlarına 3.0 ipek dikiş yerleştirilerek elde edildi. Alveoler kemik seviyesi (AKS), ataşman seviyesi (AS) ve alveolar kemik alanı (AKA) histomorfometrik yöntem ile incelendi. Receptor activator of nuclear factor kappa-B ligand (RANKL) ve Osteoprotegerin (OPG) immünoreaktivitesi ve RANKL/OPG oranları immünhistokimyasal analiz ile değerlendirildi. Bulgular: Palmatin gruplarındaki alveolar kemik alanı değerleri, EP grubuna göre istatistiksel olarak daha yüksek tespit edildi ($p<0.05$). AKS ve AS değerleri, palmatin gruplarında EP grubuna göre anlamlı olarak daha düşük bulundu ($p<0.05$). AKS, AS ve AKA değerleri için, EP-5P ve EP-10P grupları arasında anlamlı bir fark bulunmadı ($p>0.05$). RANKL / OPG oranı, deney süresi boyunca palmatin ile tedavi edilen gruplarda belirgin olarak daha düşük gözlendi ($p<0.05$). Buna ek olarak, RANKL / OPG oranına göre EP-5P ve EP-10P arasında anlamlı bir fark bulunmadı ($p>0.05$). Tartışma: Palmatinin periodontal hastalıkta alveoler kemik kaybı ve bağ dokusu yıkımı üzerine koruyucu etkisi olabilir.

Anahtar Kelimeler

Periodontal Hastalık; Palmatin; Alveolar Kemik Kaybı

Abstract

Aim: The aim of this study is to examine the effects of orally administered palmatine on periodontium in an experimental periodontitis (EP) rat model. Material and Method: A total of 40 rats were divided into 4 groups as group 1, no EP and no palmatine (CTRL); group 2, EP and no palmatine (EP); group 3, EP and 5mg/kg of palmatine (EP-5P); group 4, EP and 10mg/kg palmatine (EP-10P). Experimental periodontitis was achieved by placing 3.0 silk sutures on the cervical margins of the right mandibular first molar teeth of the rats. The alveolar bone loss, attachment loss and alveolar bone area were evaluated by histomorphometric evaluation. Immunohistochemical evaluation was used to assess the Receptor activator of nuclear factor kappa-B ligand (RANKL) ve osteoprotegerin (OPG) immunoreactivity and RANKL/OPG ratio. Results: Alveolar bone area (ABA) values in palmatine groups were statistically higher than EP group ($p<0.05$). Alveolar bone level (ABL) and attachment level (AL) values were significantly lower in palmatine groups than EP group ($p<0.05$). For ABA, ABL and AL values, no significant difference was found between groups EP-5P and EP-10P ($p>0.05$). According to the RANKL / OPG ratio, a significant decrease was observed in the palmatine-treated groups during the experimental period ($p<0.05$). In addition, there was no significant difference between EP-5P and EP-10P according to RANKL/OPG ratio ($p>0.05$). Discussion: Palmatine might have protective effects on alveolar bone loss and connective tissue destruction in periodontal disease.

Keywords

Periodontal Disease; Palmatine; Alveolar Bone Loss

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Introduction

Periodontal disease (PD) is a chronic inflammatory disease of teeth supporting tissues[1]. Although microbial dental plaque is considered as the primary etiologic factor, the onset and progression of the disease is the result of the inflammatory response of the host. PD is characterized by gingival inflammation, periodontal pocket formation, connective tissue attachment loss, and alveolar bone resorption that may result in tooth loss [2-4].

The osteoclast-derived bone resorption pathway is related to the interaction of the TNF superfamily. As members of this family, Nuclear factor-kB ligand receptor activator (RANKL); its receptor, RANK; and the decoy receptor, osteoprotegerin (OPG). RANK is a cell surface receptor of osteoclast precursor cells and osteoclasts[5]. RANKL is a potent osteoclastogenic factor synthesized by osteoblasts, fibroblasts, lymphocytes, and osteocytes[5,6]. By directly binding to RANK, it leads to osteoclast differentiation and activation, and initiates bone destruction[6,7]. RANKL activity is regulated by OPG produced by bone marrow stromal cells, osteoblasts, and osteocytes[5-7]. OPG binds to RANKL and inhibits RANKL + RANK composition, thus inhibiting osteoclastogenesis and bone destruction[7].

Palmatine is a yellow, protoberberine alkaloid derived from some plants like *Cortex phellodendri* and *Rhizoma coptidis [8]* and is structurally similar to berberine inhibiting bone destruction in osteoporotic models. Palmatine is used in the treatment of hypertension, acute and chronic inflammation, and liver related diseases[9]. By affecting serum RANKL and OPG levels, palmatine is believed to reduce bone destruction due to the anti-resorptive effect[10].

This study is based on the hypothesis that the antiresorptive effect of palmatine may reduce alveolar bone destruction in the periodontal inflammation process and may have a protective effect on periodontal inflammation. The purpose of this study was to investigate the effects of orally administered palmatine on periodontium by evaluating the alveolar bone loss, attachment loss and alveolar bone area and changes in the RANKL/OPG ratio in a ligature-induced experimental periodontitis (EP) model in rats.

Material and Method

Experimental Design:

Study protocol was approved at Bulent Ecevit University Animal Experiments local ethics committee number: 2016-01-06/01. 40 male Sprague-Dawley (SD) rats aged 6-8 weeks (200-250 grams) were used. The weekly weight checks were followed to prevent the rats from not feeding enough. The rats were housed in separate plastic cages in a room with temperature control and a standard lighting cycle, and fed with sufficient water and food. The rats were divided into 5 groups. Accordingly, the groups were organized as; group 1, no EP and no palmatine delivery (control-CTRL); group 2, EP and no palmatine delivery (EP); group 3, EP and 5mg/kg of palmatine (EP-5P); group 4, EP and 10mg/kg palmatine (EP-10P).

Induction of experimental periodontitis:

General anesthesia was administered with intramuscular injection of 3 mg / kg Xylazine HCl (Rompuns; Bayer Leverkusen, Germany) and 35 mg / kg Ketamine HCl (10% Ketasol; Richter Pharma AG, Wels, Austria) to the rats scheduled to develop EP. EP was accomplished by placing 3.0 silk sutures on the cervical margins of the right mandibular first molar teeth of the rats in groups 2, 3 and 4. The experimental period ended with the sacrificing rats under general anesthesia.

Administration of palmatine:

Palmatine was applied for 15 days starting the day before the insertion of the ligatures in group 3 and group 4 in doses of 5 mg/kg and 10 mg/kg, respectively. Palmatine was given by gavage once a day by dissolving in distilled water.

Histological analysis:

10% buffered formalin was used to fix the right side of the removed mandibles. For decalcification of the samples 10% formic acid (Merck Millipore Corporation; Darmstadt, Germany) at room temperature (pH 7.2) was used. Next the samples were embedded in paraffin (Agar, Cambridge, UK). Serial paraffin sections were taken mesiodistally along the mandibular first molar using a rotary microtome (Leica RM 2135; Leica Instruments, Nussloch, Germany). Three sections were selected and were stained with hematoxylin and eosin (H&E) representing the center of each tooth. The alveolar bone level (ABL) of the first molar tooth, alveolar bone area (ABA) and the attachment level (AL) were evaluated in all sections. A light microscope (BX50 research microscope, Olympus, Tokyo, Japan) was used for the histometric analysis. Images of the sections are digitized using a camera (DP26 Digital Camera, Olympus, Tokyo, Japan). The evaluations were performed with the OLYMPUS DP2-BSW application software. The ABL of the first molar tooth were measured from the cemento-enamel junction (CEJ) to alveolar bone crest (ABC). The furcation area was detected using an imaginary line located between the roots. The sum of the trabecular bone area and the bone marrow area were considered as the alveolar bone area. Attachment level (AL) was determined by calculating the area between the CEJ and the most coronal portion of connective tissue attachment (CTA) to cementum (Figure 1). All analysis were performed by a calibrated examiner who is blinded to the study design with respect.

Immunohistochemical analysis:

A streptavidin-biotin complex (Abcam, Cambridge, MA, USA) was used for immunohistochemical evaluation. The polyclonal anti-RANKL and anti-OPG (Boster Biological Technology Ltd., Fremant, USA) were used for detection. The endogenous peroxidase was treated with 3% hydrogen peroxide for 10 minutes at 25 ° C. Sections were incubated with the primary antibody overnight at 4 ° C in a humidified chamber. Subsequently, sections were washed with 1% PBS / BSA and incubated with biotinylated secondary antibodies (Abcam, Cambridge, Mass., USA) for 60 min at room temperature. HRP / AEC chromogen kit (Abcam, Cambridge, Mass., USA) was used and sections were stained with Mayer's hematoxylin (Sigma, Saint Louis, USA). Positive cells were painted brown. Immunoactivity was scored using HScore[11]. Immunohistochemical density scoring was evaluated as 0 (none), 1 (weak), 2 (moderate), 3 (intense). The following formula was used in the calculation: HSCORE = ΣPi (i + 1). According to the formula, i represents intensity scores, Pi percentage of stained cells, and 1 is the correction factor.

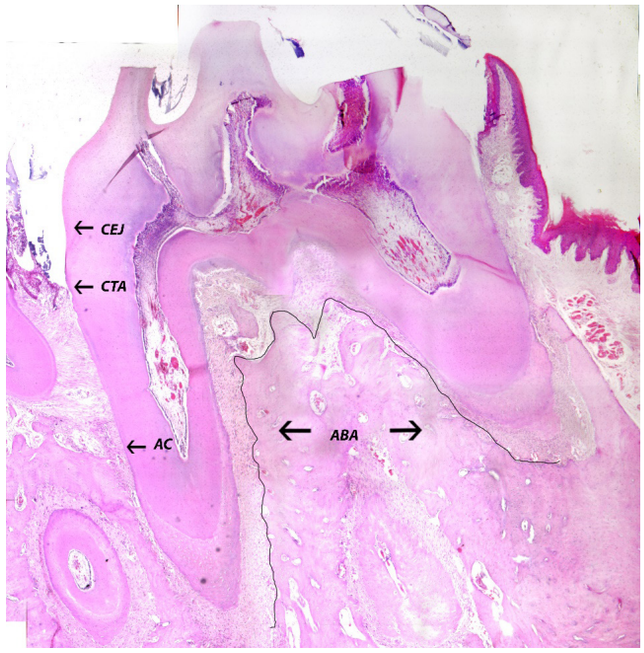

Figure 1. Histologic presentation of alveolar bone area, alveolar bone level, and the attachment level.
Sections from the mesio-distal aspects throughout the mandibular first molars and the reference areas for histomorphometric analysis (H&E, 4×). CEJ: cemento-enamel junction, CTA: connective tissue attachment, AC: alveolar crest, ABA: alveolar bone area.

Statistical analysis:

Statistical analyzes were performed using a computer software (SPSS 19.0). One-way Anova test (posthoc test, Tukey's) was applied for intergroup comparison for normal distribution data. Non-parametric tests were evaluated using the Kruskall-Wallis test. Data are presented as mean ± SD and the statistical significance value was accepted as $p<0.05$.

Results

All rats given palmatine were able to tolerate the alkaloid. No rats died during the experimental period. Although weight loss was seen, it did not reach the size that would affect the life functions. In EP group, alveolar bone and attachment loss were significantly higher than control group ($p<0.05$).

Histomorphometric results:

ABA, ABL and AL values are shown in table 1. ABA values in palmatine groups were statistically higher than control and EP groups ($p<0.05$). However, no significant difference was found between groups EP-5P and EP-10P, in which palmatine was administered at different doses throughout the course of the experiment ($p>0.05$). ABL and AL values were significantly lower in palmatine groups than EP group ($p<0.05$). For both ABL and AL values, no significant difference was found between groups EP-5P and EP-10P given palmatine during the study period ($p>0.05$).

Table 1. Alveolar bone area, alveolar bone level, and the attachment level among groups.

GROUPS	ABA (%)	ABL (µm)	AL (µm)
Group 1. CTRL	71.39±4.01	664.02±13.90	337,97±30.10
Group 2. EP	49.37±1.08[a]	1111.90±64.78[a]	1016,94±73.55[a]
Group 3. EP-5P	60.94±3.97[a,b]	898.00±56.30[a,b]	878,29±21.73[a,b]
Group 4. EP-10P	65.43±2.76[a,b]	849.99±8.78[a,b]	852,28±15.15[a,b]

Data are expressed as the mean ± SD
ABL: Alveolar bone level, ABA: alveolar bone area, AL: attachment level, CTRL: control group, EP: Experimental periodontitis group, EP-5P: 5 mg/kg palmatin administrated group, EP-10P: 10 mg/kg palmatin administrated group.
[a]significantly different from CTRL group ($p<0.05$)
[b]significantly different from EP group ($p<0.05$)

Immunohistochemical results:

Table 2 shows the OPG and RANKL immunoreactivity scores. There was a significant difference between EP and CTRL groups in terms of OPG and RANKL scores ($p<0.05$). RANKL immunoreactivity was significantly increased in EP group when compared with control group ($p<0.05$). The RANKL immunoreactivity was found significantly lower in the palmatine treated groups compared with EP group during the experimental period ($p<0.05$). There was also a significant difference between CTRL and palmatine treated groups ($p<0.05$). However, no significant difference was found between EP-5P and EP-10P ($p>0.05$). OPG immunoreactivity was significantly decreased in the EP group when compared with control group ($p<0.05$). OPG immunoreactivity was significantly increased in palmatine treated groups than EP group ($p<0.05$). There was a significant difference between groups EP-5P and CTRL ($p<0.05$), but there was no difference between groups EP-10P and CTRL ($p>0.05$). In contrast to RANKL results, a significant difference was also found between EP-5P and EP-10P ($p<0.05$). Furthermore, no significant difference was observed between CTRL and EP-10P groups ($p>0.05$). According to the RANKL / OPG ratio, the EP group was found to be significantly higher than the CTRL group, and a significant decrease was observed in the palmatine-treated groups during the experimental period ($p<0.05$). In addition, there was no significant difference between EP-5P and EP-10P according to RANKL/OPG ratio ($p>0.05$).

Discussion

The aim of the study is to investigate the protective effect of palmatine on tissue destruction during periodontitis formation. Periodontitis is a disease characterized by tooth supporting tissue destruction by the direct effect of microorganisms in the plaque and indirectly through the immune-inflammatory host response[1-3]. Various cytokines, proteases are involved in the host response[3]. Another important system for osteoclastic activity is the RANK / RANKL / OPG system. RANK is a receptor expressed by osteoclast progenitor cells. RANKL and OPG are cytokines produced from osteoblasts and bone marrow stromal cells bound to the TNF family[5-7]. The RANKL RANK compound causes active osteoclast production whereas the OPG RANKL compound inhibits the osteo-

Table 2. The HSCORE values of OPG and RANKL immunoreactivity among groups.

GROUPS	RANKL	OPG	RANKL/OPG
Group 1. CTRL	115,81±5.59	244,61±7.73	0,47±0.03
Group 2. EP	254,84±8.99[a]	148,20±10.22[a]	1,73±0.15[a]
Group 3. EP-5P	187,61±10.00[a,b]	218,00±5.49[a,b]	0,86±0.06[a,b]
Group 4. EP-10P	176,86±4.60[a,b]	231,86±2.43[b,c]	0,76±0.02[a,b]

Data are expressed as the mean ± SD
CTRL: control group, EP: Experimental periodontitis group, EP-5P: 5 mg/kg palmatin administrated group, EP-10P: 10 mg/kg palmatin administrated group.
[a]significantly different from CTRL group ($p<0.05$)
[b]significantly different from EP group ($p<0.05$)
[c]significantly different from EP-5P group ($p<0.05$)

clast differentiation process[7]. Hence, the high ratio of RANKL / OPG is associated with the destruction process in periodontal disease. RANKL / OPG ratio was evaluated immunohistochemically for the evaluation of osteoclastic activity[7].

Animal models have advantages such as easier formation of the disease to be examined and providing important information on the pathogenesis of the disease[12]. Rat ligature method is a method in which silk suture is placed in the sulcus of the first molar teeth. As a result of plaque accumulation in the region, ulceration of the epithelium, connective tissue invasion and periodontal tissue loss are expected. It is an easy and inexpensive method[13]. Due to these advantages, SD wistar rats were used in our study and experimental periodontitis was created by ligature method. Different information about the period of experimental periodontitis formation is mentioned in the literature[11,14,15]. Previously, it was determined that ligament-induced bone destruction occurred within 15 days[16]. Therefore, the experimental period in this study was completed on day 15.

Palmatine is a protobarberine alkaloid and has many biological properties such as antipyretic, antibacterial, antioxidative and anti-inflammatory properties [17-19]. In the literature, it has been shown that palatinin has anti-resorptive effect[8,10]. It has also been shown to reduce bone resorption in osteoporotic models [10]. It is shown that palmatine plays an important role in osteoclast apoptosis via the nitric oxide synthase (NOS) system in osteoclasts [20]. Also, it is indicated that palmatine inhibits the expression of the RANKL gene in osteoblastic cells [8]. In addition, palmatine has been reported to decrease RANKL / OPG ratio by decreasing both OPG and RANKL levels [10]. This research was based on the hypothesis that palmatine would reduce tissue destruction during periodontitis formation due to the anti-inflammatory and antiresorptive effects.

A recent study has shown that doses of palmatine 1 mg / kg and 10 mg / kg are safe and do not have toxic effects [10]. So, doses of 5 mg / kg and 10 mg / kg were found suitable to use in our study and it was investigated which doses are more effective and sufficient for suppression of periodontal inflammation.

In the present study, the results were evaluated histomorphometrically and immunohistochemically. To our knowledge, this is the first study to investigate the protective effect of palmatine on bone attachment in the experimental periodontitis model. So, we cannot compare ABL and AL values with another study using palmatine. However, the bone and attachment levels in the dental periphery are the data used in the evaluation of the effects of many new biologically active substances [11]. In our results, according to the control group in EP group, ABL and AL are higher and ABA is lower. This shows that experimental periodontitis has developed. According to the histological results of our study, ABL and AL were less in the palmatine applied groups than in the EP group and ABA was found to be higher in the palmatine applied groups than in the EP group. This result indicates that palmatine has a protective effect on the periodontal inflammation formation and destruction process. We can characterize this effect as an antiresorptive effect in accordance with the literature[8,10]. We cannot mention about bone regeneration as we cannot comment for therapeutic purposes because we did not apply after experimental periodontitis; but our results suggest that palmatine reduces the severity of periodontal destruction when applied to rats during experimental periodontitis.

A recent study showed that palmatine (10 μM) significantly reduced RANKL and OPG levels in the culture supernatant of MC3T3-E1 cells and in serum and stated that the RANKL/OPG ratio also decreased depending on the concentration of palmatine [10]. Another study revealed that the inhibitory effect of palmatine on osteoclastogenesis was due to inhibition of the RANKL gene expression [8]. According to our immunohistochemical results, RANKL immunereactivities increased and OPG scores decreased in the experimental periodontitis group compared to the control group. In the palmatine treated groups, it was determined that RANKL scores decreased with respect to periodontitis group. Contrary to the previous study[10], there was an increase in OPG scores after palmatine administration in our study and most importantly RANKL / OPG ratio decreased with palmatine treatment. This can theoretically result from both the decrease in RANKL scores and the increase in OPG scores.

Another aim of our study is to examine which dose will be more effective and sufficient. In a previous study, administration of palmatine at 10 mg / kg was found to have antiresorptive effect [10]. We investigated in this study whether the dose of 5mg / kg would be sufficient to reduce periodontal destruction in an experimental periodontitis model. When the given doses of 5 mg / kg and 10 mg / kg were compared, there was no significant difference between the two doses in terms of RANKL immunoreactivity, but a significant difference was observed between 5 mg / kg and 10 mg / kg in terms of OPG immunoreactivity. However, when the RANKL / OPG ratio was evaluated, there was no significant difference between the two doses. According to the histological results of our study, no significant difference was observed when ABA, ABL and AL levels were evaluated between 5 mg / kg and 10 mg / kg palmatine given groups. As a result, oral administration of palmatine at a dose of 5 mg / kg is considered to be sufficient to reduce periodontal inflammation and destruction.

In conclusion, new biological substances are being studied for the prevention and treatment of periodontal inflammation. The clinical significance of this study is the reduction of periodontal tissue destruction by using palmatine. On the other hand, while we know that these doses are not harmful to rats, we cannot comment on the appropriate dose that should be used in humans. Clinical trials are therefore needed to determine the appropriate dose of palmatine that can be used in humans.

Ethical Responsibilities

All institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of Interest:

No potential conflict of interest relevant to this article was reported.

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