**Histopathological and biochemical evaluation of paeoniflorin administration in an experimental periodontitis model**

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**Abstract:** The purpose of this study was to evaluate the effects of administered Paeoniflorin (Pae) on periodontal tissues within an experimental periodontitis model. Forty male Wistar rats were used in this study and experimental periodontitis was created in all rats except in the control group (n = 10, first group). In the periodontitis group, experimental periodontitis was created but no other application was performed (n = 10, second group). In the other groups created experimental periodontitis, systemic Pae (n = 10, third group) or saline (n = 10, fourth group) was applied. A biochemical analysis of the gingival vascular endothelial growth factor (VEGF) levels and a histomorphometric analysis (measurements of the area of alveolar bone, alveolar bone resorption, and attachment loss) were performed. In the Pae group, the area of the alveolar bone was increased, while alveolar bone resorption and attachment loss decreased. Gingival VEGF levels increased in all groups that created experimental periodontitis and the greatest increase seen in the Pae group. Histomorphometric and biochemical analyses in this study suggest that Pae has a curative effect on periodontal tissues. However, additional studies are needed to confirm these results.

**Keywords:** experimental periodontitis, histology, herbal medicine

**Introduction**

Periodontitis is a multifactorial, chronic inflammatory disease of the periodontium caused by interactions between periodopathogens and the host’s defense system, leading to the destruction of tissues around the teeth [1,2]. In addition to the direct effects of these pathogenic microorganisms, periodontal destruction occurs from indirect mechanisms related to the host’s immune response [3]. Some changes in periodontal vascular structures have been observed during the development of periodontal disease. The various cytokines and growth factors also are effective in regulating angiogenesis, which increases in infected tissues [4-7]. Vascular endothelial growth factor (VEGF), a member of the platelet-derived growth factor superfamily, is an important glycoprotein primarily found in endothelial cells. VEGF regulates angiogenesis, increases vascular permeability, stimulates endothelial cell proliferation and differentiation, and induces migration of endothelial cells. This regulation occurs in both physiologic and pathologic events such as wound healing, ischemia, cancer, and inflammation [8-11]. A relationship exists between periodontal disease and VEGF; however, there are conflicting results regarding the role of VEGF in the pathogenesis of periodontal disease [6,7,11,12]. One study noted that VEGF may be associated with both periodontal tissue remodeling and tissue destruction [7]; however, Çetinkaya et al. (2007) found that VEGF expression was associated with recovery from periodontal disease rather than tissue destruction [12]. Paeoniflorin (Pae) is a monoterpene glycoside isolated from the roots of the *Paeonia lactiflora* plant and has been used as a medicinal herb in traditional Chinese medicine [13]. It is a significant bioactive component of the in total glycosides of the extract obtained from the roots and is responsible for their biologic effects [13]. Pae has been shown to have anti-inflammatory and immunomodulatory effects and has been used for many years in the treatment of autoimmune/inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus, diabetic nephropathy, and Sjögren syndrome [13-15]. Pae suppresses bone resorption and, by stimulating bone formation, has a positive effect on bone metabolism and remodeling [16,17]. Additionally, Pae has been shown to suppress excessive osteoclastic activity [16,18]. Pae has been approved by China’s State Food and Drug Administration as a disease-modifying drug in the treatment of rheumatoid arthritis [19]. Given its systemic effects, Pae might be a treatment for metabolic syndromes and systemic diseases associated with bone loss.

The hypothesis of this study was that Pae is effective as a new curative agent in the treatment of periodontal diseases by regulating the host response. Although many studies have evaluated the anti-inflammatory effects of Pae on multiple diseases, there is possibly only one recent study that has examined the effects of Pae on hard and soft tissues in periodontal disease [20]. The present study was based on the hypothesis that Pae has a curative effect on bone attachment and loss. This study was designed to evaluate the anti-inflammatory activity of systemic Pae on periodontal tissue using histomorphometric and biochemical analyses of rats with experimental periodontitis.

**Materials and Methods**

**Subjects and design**

Forty male Wistar albino rats (weighing 150-250 g) were used in this study. The animals were kept in cages with controlled environmental conditions (22-1°C; 50% humidity) with light and dark cycles (12:12 h). They were given food and water *ad libitum*. All protocols were approved by the Ondokuz Mayıs University’s Ethical Committee of Animal Research (protocol no: 2014/27).

The subjects were divided randomly into four equal groups. Silk ligatures were tied around the mandibular first molars of all rats except the control group. The ligatures were kept in place for 15 days to create an experimental periodontitis model [11,21].

The groups were treated as follows:

- **Group 1**: control group (healthy and without any treatment) (n = 10).
- **Group 2**: periodontitis group. Sacrification was performed after the creation of experimental periodontitis (n = 10).
- **Group 3**: Pae group. After the formation of experimental periodontitis, ligatures were removed and systemic Pae was applied per os for 7 days. Subsequently, sacrification was performed (n = 10).
• Group 4: saline group. After the formation of experimental periodontitis, ligatures were removed and systemic saline was applied for 7 days (0.2 mL). Subsequently, sacrifice was performed (n = 10).

In Pae group, Pae was applied systemically at a dose of 100 mg/kg dissolved in 0.2 mL saline solution. This solution was mixed by vortexing and applied by gavage [22]. The saline group was a control group for the Pae group.

Histomorphometric analyses
Following sacrifice, left mandibular samples were fixed in 10% neutral formalin and decalcified for 14 days for histomorphometric examination (in 8% formic acid). Then, the samples were washed for 12 h and routine histologic evaluation was performed. Sections 5-μm thick were taken from the first molar tooth in a mesiodistal direction using a rotary microtome and stained with hematoxylin & eosin (H&E). Three different histometric measurements were obtained from each sample in the mesiodistal sections, and the arithmetic mean of the measurements was calculated. In the H&E-stained sections, artificial standard limits were established. Reference volumes of the anatomical structures were identified to determine changes in the periodontal tissues. A light microscope (Olympus BX50 research microscope; Tokyo, Japan) were used for performing histomorphometric analysis and a monitor with a camera apparatus (Olympus DP26 digital camera) were used for transferring the images, the following parameters were evaluated [23]:

1) area of alveolar bone (AA): alveolar bone area / furcation area (ratio, %);
2) alveolar bone resorption (ABR): distance from the cemento-enamel junction to the alveolar bone crest (µm);
3) attachment loss (AL): distance between the cemento-enamel junction and the most coronal extent of the connective tissue attachment (µm). Designation of the reference areas for histomorphometric analysis is shown in Fig. 1.

Biochemical analysis
Gingival tissues surrounding the right mandibular first molar teeth were removed using excisional biopsy. The samples were placed into Eppendorf tubes and stored at −80°C until biochemical analysis.

Tissue extracts were performed according to kit procedures. Samples were washed with phosphate buffered saline (0.01 mol/L, pH 7.0-7.2) to remove debris and then dried. The tissue was weighed before being cut into small pieces with a scalpel. Tissue pieces were placed in a thick glass tube and dissolved with 1 mL phosphate buffered saline containing protease inhibitor (5 μg/mL aprotinin, 1 mM EDTA) to give 10 mg tissue/mL. The dissolved samples were sonicated five times for 30 s each time using a UV2200, Bandelin Elc. device. Following sonication, the suspension was centrifuged and the supernatant was removed. The supernatant was stored at −20°C until enzyme-linked immunoabsorbent assay (ELISA) analysis was performed. The VEGF levels of rats’ gingival tissue were analyzed in each 150-μL sample by ELISA at 450-550 nm according to the kit manufacturer’s protocol (Thermo Fisher Scientific, ERVEGFACL; Schwerte, Germany).

Statistical analysis
Statistical analysis was performed using SPSS 21.0 Packet Data Program (SPSS 21.0 Software Package Program Inc., Chicago, IL, USA). The data were tested for normality using the Shapiro-Wilk test. All group comparisons were performed using the Kruskal-Wallis test as analysis of the data did not show a normal distribution. Pairwise comparisons of the groups with statistically significant differences were analyzed using a Bonferroni-adjusted Mann-Whitney U test. A P < 0.008 was considered statistically significant in the analysis of the six possible pairwise comparisons.

Results
Clinical findings
Pae was tolerated by all animals, and no complications occurred following periodontitis formation and systemic drug administration. By day 15, significant alveolar bone and AL were detected clinically and radiographically in the groups created experimental periodontitis compared with the control group. Radiographic images of the groups are shown in Fig. 2.

Histomorphometric findings
AA (%), ABR (µm), and AL (µm) values are listed in Table 1. Histomorphometric measurements are found in Fig. 3, and the furcation area of the first molar tooth in the mesiodistal sections are noted in Fig. 4.

AA in the furcation area was significantly greater in the Pae group than in the saline group (P = 0.000). There was no statistically significant difference between the saline and periodontitis groups (P = 0.393). There was a statistically significant decrease in AL in the Pae group compared with the periodontitis and saline groups (P = 0.000). Among the groups created experimental periodontitis, the ABR was the lowest in the Pae group (P = 0.004).

Biochemical findings
Table 2 presents gingival VEGF levels of the study groups. Gingival VEGF levels were significantly higher in the groups created experimental periodontitis than in the control group (P = 0.000). Gingival VEGF levels were also significantly higher in the Pae group than the periodontitis and saline groups (P = 0.000).

Discussion
This study was performed to research the anti-inflammatory effect of systemic Pae administration on the periodontium of rats with experimental
periodontitis using histomorphometric and biochemical analyses.

Although various techniques have been used to generate experimental periodontitis in rats, endotoxin injection and ligation of teeth are the most commonly used techniques [24]. When periodontitis is induced by endotoxin injection, periodontal disease is not induced directly by periodontal pathogens but acute and severe inflammation occurs [24,25]. It has also been noted that additional trauma is present in the molar region following endotoxin injection because it requires continuous injection throughout the experimental period [26]. However, placement of ligatures around the teeth allows plaque build-up causing ulceration of the sulcular epithelium. This study used an experimental periodontitis model generated by ligature, which is a simple and reliable technique for evaluating the progression of periodontal disease in rats [27-29]. This model causes plaque accumulation around the ligature resulting in inflammatory cell recruitment and production of chemical mediators. Ultimately this leads to the destruction of periodontal hard and soft tissues in a manner similar to the progression of human periodontitis [30]. The structural, immunological, and microbiological properties of rat periodontal tissue are similar to that of human molars [31-33].

Pae, a root extract of *Paeonia lactiflora*, is widely used in the treatment of chronic inflammatory diseases. Pae has been administered to rats for treatment with adjuvant arthritis (25, 50, 100 mg/kg/day; 7 days), allergic contact dermatitis (35, 70, 140 mg/kg/day; 7 days) and experimental periodontitis (30, 60 mg/kg/day; 7 days) (Respectively; at various doses, lengths of time) [20,22,34]. The dose and duration of Pae (100 mg/kg/day for 7 days) used in the present study was chosen based on studies evaluating the effects of Pae on inflammatory diseases. These studies noted that this dose has an anti-inflammatory effect without any side effects [20,22,34].
1. AlMoharib HS, AlMubarak A, AlRowis R, Geevarghese A, Preethanath RS, Anil SJ (2014) The authors report no conflict of interest related to this study. This study was supported by the Scientific Research Fund of Ondokuz Mayıs University. Acknowledgments

2. This study suggests the need for further studies.

3. VEGF has been shown to contribute to the spread of inflammation by increasing vascular permeability in studies noting increased VEGF levels in periodontal disease [5,6]. VEGF has also been found to be involved in the remodeling, repair, and regeneration of periodontal tissue [12]. According to the findings of the present experimental study, the highest levels of VEGF in the Pae group support studies suggesting that VEGF is more closely related to the healing stage of periodontal disease rather than to the tissue destruction stage [12]. The changes in gingival tissue VEGF levels support the histomorphometric findings in the present study, and Pae may be considered to be associated with periodontal tissue healing when this process and dose are used. Taken together, the results of the present study suggest the need for further studies.

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Conflict of interest

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

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