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ORIGINAL ARTICLE

Protective effects of ginkgo biloba extract on ligature-induced periodontitis in rats

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Abstract

Objective. The aim of this study was to test the hypothesis that the systemic administration of extract of Ginkgo biloba (EGb) would prevent excessive tissue destruction in ligature-induced periodontitis in a rat model. ***Materials and methods.*** Thirty-two male Wistar albino rats were used in the current study. The rats were randomly divided into four groups of eight rats each: (1) non-ligated treatment (NL) group, (2) ligature-only (LO) group, (3) ligature plus GB28 (28 mg/kg, daily for 11 days) group and (4) ligature plus GB56 (56 mg/kg, daily for 11 days) group. ***Results.*** Measurement of alveolar bone loss in the mandibular molar tooth revealed significantly lower bone loss values in the LO group compared to groups NL, GB28 and GB56 ($p < 0.05$). ***Conclusion.*** The present results are the first data which suggests that host response in periodontitis can be modified by EGb administration. EGb minimized progression of periodontal disease.

Key Words: *antioxidant, experimental periodontitis, ginkgo biloba extract, host modulation therapy*

Introduction

Periodontitis is a well-documented example of leukocyte-mediated bone loss and inflammation that has pathogenic features, similar to those observed in other inflammatory diseases [1]. All forms of inflammatory periodontal disease are associated with the accumulation of B and T lymphocytes. Added to these, monocytes and neutrophils can also be pronounced as chronic inflammation and, if left untreated, irreversible tissue damage can occur in the bone and soft tissue surrounding teeth, leading to tooth mobility and ultimately the loss of teeth [2]. The production of reactive oxygen species is the essential pathogenic mechanism for periodontal diseases associated with phagocytosis as the host defense against bacterial pathogens [3,4]. However, when reactive oxygen species overwhelm the cellular antioxidant defense, further tissue injury becomes inevitable, which ultimately results in increased oxidative stress [5,6].

Although the role of microorganisms in the etiology and pathogenesis of periodontal disease is evident, studies have revealed that the protective and/or destructive effects of host response is one of the determinant factors in the severity of periodontal diseases and disease outcome [7].

Host modulation means modifying or modulating destructive or damaging aspects of the inflammatory host response which develops in the periodontal tissues as a result of chronic challenge presented by the subgingival bacterial plaque [8]. While pharmacological approaches to manage periodontal diseases were anti-infective in nature in the past, for the last decades periodontal scientists have produced compelling evidence for the potential benefit of chemotherapeutic drugs which modulate the host response. Based upon animal models of disease and early clinical trials, there appears to be great promise for the therapeutic application of non-steroidal anti-inflammatory drugs (NSAIDs) [9], anti-cytokine drugs [8], bone resorption uncouplers

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[10] and recently antioxidants [11,12] for the management of periodontal diseases.

Antioxidants, many of which are released locally at sites of inflammation by polymorphonuclear leukocytes (PMNLs) and/or other cells, can provide protection against reactive oxygen species (ROS). In healthy organisms, the balance is maintained by the interaction of oxidants and antioxidants. Under pathological conditions, the balance may be directed towards the oxidative side [13,14]. In recent years it has been proposed that dietary supplements with an antioxidant activity could be responsible for the reduction of chronic diseases [12]. Research supplies a hypothetical mechanism by which antioxidant substances may be reducing the risk of periodontal destruction through the inhibition of oxidative damage [15]. Such data raises the following problem of whether supplementation of these dietary supplements emerges as being useful in the primary prevention of tissue damage in periodontitis.

Ginkgo biloba leaf extract (EGb) is among the most widely sold herbal dietary supplements in the US [16]. This plant, which has its origin in China, Japan and Korea, has been used as a food as well as medicine for a long time. Phytochemical studies have shown a standard composition of substances in this plant comprising 24% of ginkgo-flavone glycosides (flavonoids) (kaempferol, quercetin and ishorhamnetin), 6% of terpenoids (ginkgolides and bilobilides) and less than 5 ppm of ginkgolic acid [17]. Its purported biological effects include: scavenging free radicals [18]; lowering oxidative stress [19]; reducing neural damages [20]; reducing platelets aggregation [21]; anti-inflammation [22]; anti-tumor activities [23]; and anti-aging [24]. However, its use in dentistry has not started yet; EGb is widely used in medicine and stil has been the subject of several studies. To the best of our knowledge, until now the effect of EGb on periodontium has been evaluated in only one study by Lucinda et al. [25] by the radiographic assesment of mandibular osteoporosis in EGb administered rats. However, the effect of EGb on periodontitis and periodontal tissues still remains unclear.

The aim of this study was to test the hypothesis that the systemic administration of EGb would prevent excessive tissue destruction in ligature-induced periodontitis in a rat model, making a histomorphological assessment.

Materials and methods

Thirty-two male Wistar albino rats (300 10 g), were used in the current study. The animals were housed under standard conditions, and the room was maintained on a 12-h light–dark cycle at ~ 25°C. The animals had free access to water and food *ad libitum*. The experimental procedure was approved by the Animal Ethics Committee of Cumhuriyet University, School of Medicine.

The rats were randomly divided into four groups of eight rats each: (1) non-ligated treatment (NL) group, (2) ligature-only (LO) group, (3) ligature plus GB28 (28 mg/kg, daily for 11 days) group and (4) ligature plus GB56 (56 mg/kg, daily for 11 days) group.

Doses were chosen according to the results reported by Lucinda et al. [25]. General anesthesia was administered using ketamine (Eczacıbasi Ilac Sanayi, Istanbul, Turkey) (40 mg/kg). In order to induce experimental periodontitis, a 4/0 silk suture (Dogsan Sanayi, Istanbul, Turkey) was placed and knotted submarginally by the same operator, around the gingival margin of right mandibular first molars of the rats. The sutures were checked after application and lost or loose sutures were replaced. In the GB groups daily systemic treatment with EGb (Tebokan, Abdi İbrahim Pharmaceutical Co., Istanbul, Turkey) was continued by gastric feeding, at a rate of 28 mg/kg/day and 56 mg/kg/day until the sacrifice of all animals on the 11th day; the rat mandibles were then separated from muscle and soft tissue, keeping the attached gingiva intact with the bone. The right mandibules were used for histomorphometric and histologic analyses.

Measurement of alveolar bone loss

All animals were examined by means of morphometric measurements of alveolar bone loss. The examiner (H. Özdemir) was masked regarding the treatment. After a gingival dissection was performed, the mandibles were de-fleshed and stained with 1% aqueous methylene blue solution (Merck, Rahway, NJ) to identify the cement–enamel junction (CEJ). The alveolar bone height was measured under a stereomicroscope (Leica Microsystems, Wetzlar, Germany) (40× magnification) by recording the distance from the CEJ to the alveolar bone crest. For evaluating average alveolar bone height, three points were measured on the buccal and lingual parts. The average alveolar bone height was calculated for each tooth.

Histopathological evaluation

Histological evaluation was carried out in a masked manner toward clinical data and treatment regimen by a single pathologist (H. Özer). The specimens were fixed in a 10% neutral buffered formalin solution and de-mineralized in an aqueous 10% formic acid solution. These specimens were then dehydrated, embedded in paraffin and sectioned along the molars in a mesiodistal plane, for Hematoxylin & Eosin staining as described by Toker et al. [26]. Light microscopy (Eclipse E 600, Nikon, Tokyo, Japan) assessment was performed on the two sections with a thickness of 6 μm, corresponding to the buccal and lingual areas

between the first and second molars where ligatures had been placed.

The areas of alveolar bone and inter-dental septum were analyzed under light microscopy, considering parameters including inflammatory cell infiltration (ICI) of the periodontal tissues, existing resorption lacunae (osteoclast surfaces), osteoblastic activity (forming surfaces), and the number of osteoclasts. ICI was determined by semi-quantitative scoring as no visible ICI (0), slightly visible ICI (1) and dense ICI (2). Osteoclasts were counted based on their morphology. In addition to the numbers, osteoclast morphology was used to define the qualitative characteristics of the cells: the absence of osteoclasts was scored as 0, osteoclasts with their usual ruffled borders were scored as 1, and osteoclasts that lacked ruffled borders and exhibited more regular cell margins were scored as 2.

The presence of osteoblastic activity (e.g. 'forming surfaces') was determined by the visibility of active bone formation surfaces that were circumscribed by osteoid and cuboidal osteoblasts. If they were not visible it was scored as 0, mild-to-moderate visibility was scored as 1 and existing osteoblastic activity with a dense mass was scored as 2.

Statistical analysis

The statistical analysis was performed using a commercially available software program (SPSS 14.0, SPSS Inc., Chicago, IL). The ratios of the presence of ICI, osteoclast morphology and osteoblastic activity were analyzed with a chi-square test. Osteoclast numbers and alveolar bone loss were analyzed with an ANOVA test, followed by a Tukey test for pair-wise comparisons. Data were presented as mean ± SD or a percentage as appropriate. p-values < 0.05 were considered statistically significant.

Results

The animals did not show any obvious signs of systemic illness throughout the study period. Weight loss was <10% in LO, GB28 and GB56 groups on day 11. Representative photos of the alveolar bone loss in the mandibular first molar are shown in Figure 1.

Measurement of alveolar bone loss in the mandibular molar tooth revealed significantly lower bone loss values in the LO group compared to groups NL, GB28 and GB56 ($p < 0.05$) (Figure 2). The alveolar bone loss in the NL group was less than the groups GB28 and GB56, but the differences were not statistically significant ($p > 0.05$).

The ratio of presence of ICI score 2 in group LO was significantly higher (100% vs 37.5%, 25% and 12.5%) than those of groups NL, GB28 and GB56 ($p < 0.05$) (Figure 3).

Figure 4 presents osteoclast number of all the groups. Osteoclast number of the LO group was significantly higher than those of groups NL, GB28 and GB56 ($p < 0.05$) (Figure 5). The ratio of presence of osteoclast morphology score 1 in the

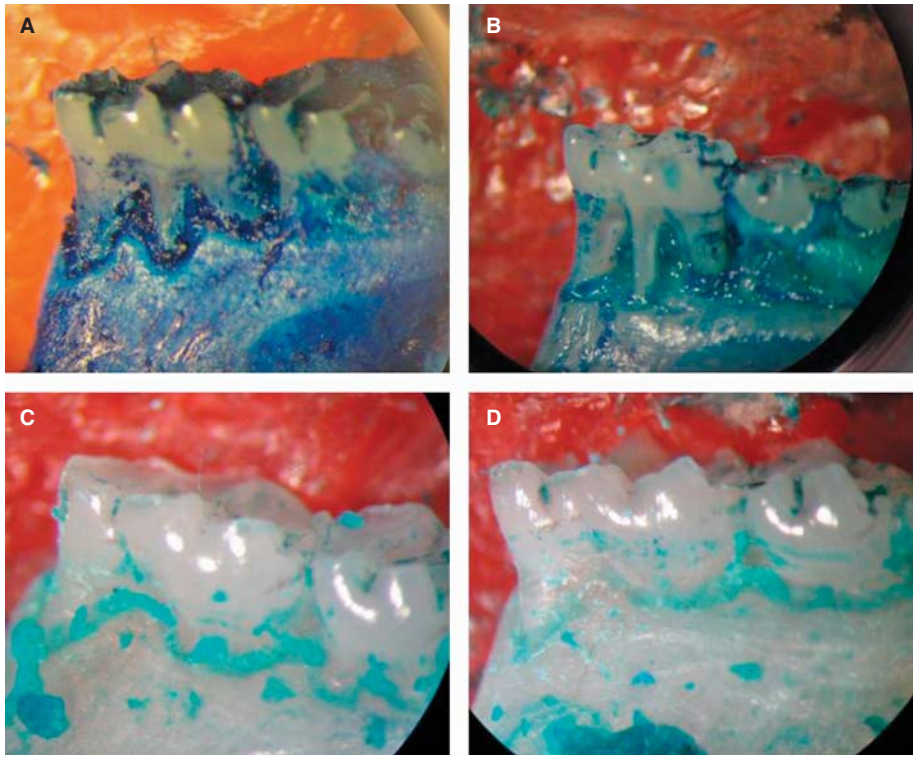

Figure 1. Representative images of the alveolar bone loss in the mandibular first molar in the (A) NL, (B) LO, (C) GB28 and (D) GB56 groups.

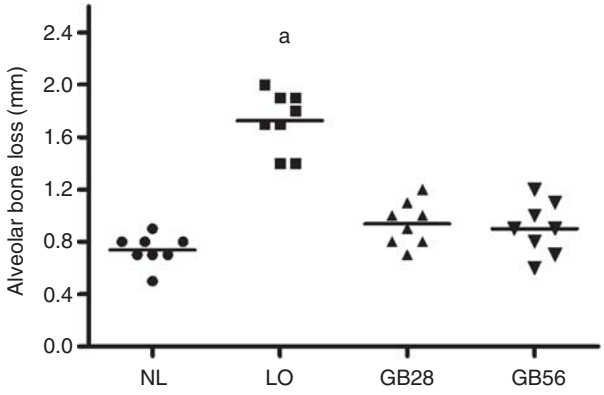

Figure 2. Mean alveolar bone loss in the NL, LO, GB28 and GB56 groups. [a]Statistically significant difference ($p < 0.05$) vs the NL, GB28 and GB56 groups. Lines = mean values.

LO group was significantly higher (100% vs 87.5%, 25% and 37.5%) than those of groups NL, GB28 and GB56 ($p < 0.05$).

The ratio of presence of osteoblastic activity score 3 in groups GB28 and GB56 was significantly higher (62.5% and 75% vs 37.5% and 25%) than those of groups NL and LO ($p < 0.05$) (Figure 5) .

Discussion

In this study we investigated the effect of EGb 28 and 56 mg/kg on alveolar bone loss in experimental periodontitis in a rat model. EGb reduced osteoclast accounts, degree of inflammation and induced osteoblastic activity. The results of the current study support the research hypothesis that the systemic administration of EGb would prevent excessive tissue destruction in ligature-induced periodontitis in a rat model.

The only study concerning the effect of EGb on jawbones of the rats, Lucinda et al. [25] reported recovery of the periodontal bone support and an increase in the mandibular cortical thickness on glucocorticoid-induced osteoporosis. However, our results are consistent with the results of that study

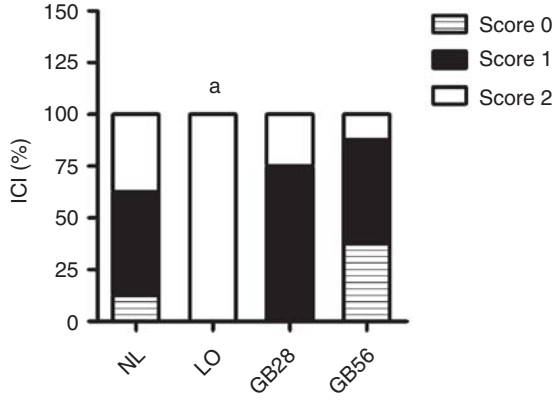

Figure 3. Frequency (%) of different inflammatory cell infiltration in the study groups. [a]Statistically significant difference ($p < 0.05$).

as regards preventive effects of EGb on periodontal bone destruction; osteoporosis and periodontitis are different diseases in nature. To the best of our knowledge it can be concluded that the current study is the first one evaluating the effects of EGb on an experimental periodontitis model.

The experimental model of ligature-induced periodontitis, such as dietary manipulation, introduction of pathogenic micro-organisms [27], is a model widely used in experimental periodontal research and mimics periodontitis in humans [28,29]. As reported by Guimaraes et al. [29], ligatures induce an increase in infiltration of inflammatory cells and production of chemical mediators which leads to the degradation of tissues surrounding the teeth of the rat, confirming that the intense recruitment of leukocytes, observed in the progression of periodontal inflammation, contributes to the destruction of periodontal tissues. Excessive recruitment and activation of leukocytes in the periodontium contributes to the progression of periodontal disease and the destruction of periodontal tissue [30]. In our study, ligature placement on the first molar tooth caused a significant amount of bone loss. Furthermore, De Lima et al. [31] demonstrated that inflammatory cells, including osteoclasts and lymphocytes, appeared beneath the ligature; significant alveolar bone loss was induced after 7 days, reached a maximum between days 7–11 and declined on the 14th day. These data support Sallay et al. [32], who proposed a maximum bone loss on the 9th day after ligature placement. In our study placement of ligature around the first molar teeth caused a significant amount of bone loss at 11th days. However, there are inherent limitations for every animal model of a human disease. Molars in rats are similar in anatomic configuration and structure to humans, but the molars of rats are smaller, so it was difficult to perform any sort of periodontal treatment [33]. A further limitation of the current experimental model is that the induced periodontitis follows an acute course, during which tissue trauma and adjacent microbial accumulation accelerates the destructive process. Such pathways of acute inflammation are likely to differ from chronic periodontitis [34]. There is, however, a ligature model, which is useful for evaluating bone loss in animal models. The technique used in order to measure the alveolar bone loss in the current study was the protocol which has been used by a number of authors [26,34].

The choice of EGb doses was based on the results reported by Lucinda et al. [25], which suggested a protective effect on periodontal destruction in osteoporotic rats with a dose of 28–56 mgs of EGb/kg/day. Despite possible side-effects induced by EGb in humans [35], in the present study there were no significant clinical changes within the current doses in rats.

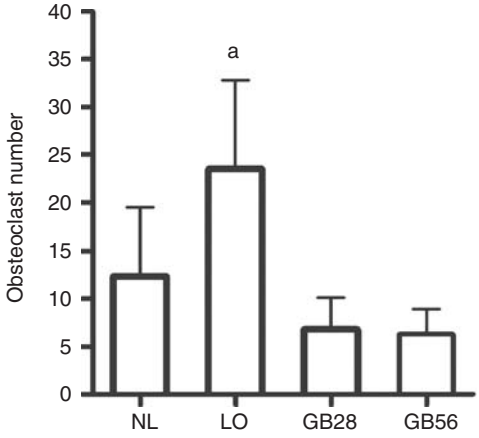

Figure 4. Osteoclast numbers in the study groups. [a]Statistically significant difference ($p < 0.05$).

In this study, alveolar bone loss was measured in order to measure the distance between alveolar bone crests and the cemento–enamel junction. The results of this study revealed that higher bone loss occurred in the LO group than in the GB and NL groups. No statistically significant difference was found regarding alveolar bone loss between the NL and GB groups. The study also revealed that osteoclastic activity and ICI was higher in the LO group than in other groups. No statistically significant difference was observed regarding ICI and osteoclast numbers between the NL and GB groups. Besides there was no statistically significant difference regarding osteoclast accounts, degree of inflammation and induced osteoblastic activity between GB28 and GB56 groups. These morphological and histological results showed that systemic administration of both 28 and 56 mg/kg of EGb diminishes periodontal inflammation and alveolar bone loss in experimental periodontitis.

Although the exact mechanism of etiopathogeny and pathophysiology of periodontitis is not clear, several lines of evidence implicate that ROS may play an important role in the pathogenesis of periodontitis [36-38]. It has been suggested that as a result of stimulation by bacterial antigens, PMNs produce and release a large quantity of ROS, culminating in heightened oxidative damage to gingival tissue, periodontal ligament and alveolar bone [39]. ROS

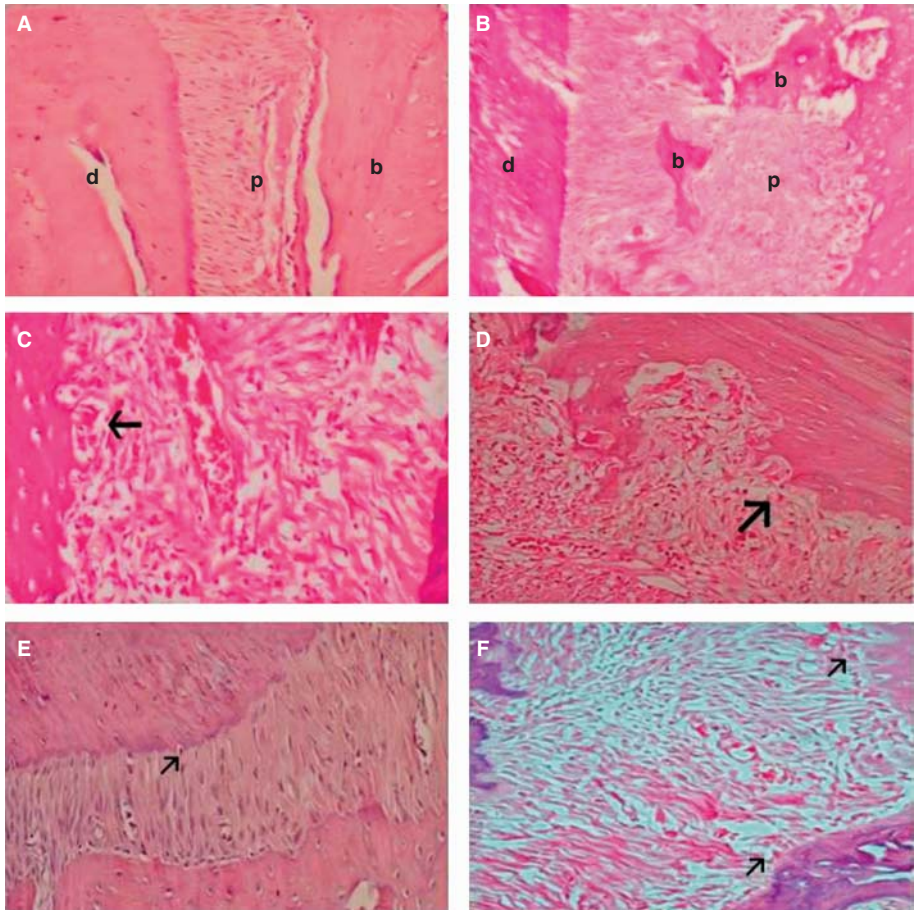

Figure 5. Histopathology in mandibular first molar tooth in all groups. (A) Normal mandibula showing alveolar bone (b), periodontal ligament (p), and dentin (d). (B) Mandibula in the LO group after 11 days of periodontitis. (C) and (D) Mandibula in the GB28 and GB56 groups after 11 days of periodontitis showing increased osteoblastic activity (arrow). (E) and (F) Mandibula after 11 days of periodontitis treated with GB (28 and 56 mg/kg), showing reduction in osteoclast number, respectively. (Hematoxylin and eosin; original magnification: A and B, ×25; C, D, E and F, ×50).

are active in the depolymerization of extracellular matrix components, lipid peroxidation, oxidation of enzymes such as anti-proteases, induction of pro-inflammatory cytokines and DNA damage [36-40].

In the current study histological data demonstrated that EGb produced a significant decrease in periodontitis-induced alveolar bone loss, osteoclast accounts and inflammatory cell infiltrations. In addition, an increase in osteoblastic activity was detected. Lucinda et al. [25] reported that the protective effects of EGb on periodontal tissues may be related to its estrogenic activity by inhibiting resorption and promoting osteogenesis. The results of the current study showed that the preventive effects of EGb on periodontal destruction observed here may be explained by not only its estrogenic activity but its antioxidant [19] and anti-inflammatory [22] properties. These effects appear to be attributed to the combined actions of ginkgolides and flavonoids. Flavonoids in EGb reportedly inhibit cyclo-oxygenase and lipooxygenase that are involved with arachidonic acid metabolism. Cyclo-oxygenase activity produces thromboxane A2, a potent platelet aggregator, and lipoxygenase is concerned with the formation of leukotrienes, the substances associated with inflammation [35]. The flavonol glycosides and proanthocyanidins have free radical-scavenging activity and thus may play a protective role in improving conditions resulting from oxidative stres [41]. Scavenging superoxide, hydroxyl and peroxyl radicals and nitric oxide may affect signal transduction [42]. Terpenes except ginkgolide A are superoxide (O_2^-) scavengers [43] and flavonoids contain many hydroxyl groups (-OH) and functional groups reducing these hydroxyl groups can play a direct antioxidant role in eliminating free radicals [41]. Flavonoids are also excellent hydrogen-atom donors and can perform hydrogen oxidation to eliminate free radicals. Besides, the antioxidant activity of flavonoids could be improved by creation of uniform electronic spin density distribution in the semiquinone free radicals, and chemical modification to increase the number of phenolic hydroxyl or hydrogen bonds in the molecule [35].

In a previous study, Emerit et al. [44] reported that the clastogenic factors in the plasma of persons irradiated accidentally or therapeutically were suppressed by EGb. Lee et al. [45] demonstrated that EGb blocked serotonin stimulated proliferation of bovine pulmonary artery smooth muscle cells as well as Chinese hamster lung fibroblasts (CCL−39) by scavenging O_2.

Çanakçı et al. [46] reported the excessive production of ROS by activated polymorphonuclear leukocytes in chronic inflammation in periodontitis may lead to premature oxidative damage of the mtDNA. Some researchers suggest that EGb may have protective effects on mitochondrial DNA (mtDNA) damage induced by free radicals [47,48].

Serum levels of assessed agents and serum metabolites can be analyzed for detailed investigation of antioxidant agents in periodontitis studies. In this study we only evaluated changes in alveolar bone levels clinically and histopathologically. So, further follow-up studies are needed for further evaluation of the effect of EGb on periodontal tissues and mechanisms of action.

Conclusion

The present results are the first data which suggests that host response in periodontitis can be modified by EGb administration. EGb-minimized progression of periodontal disease and follow-up studies are needed to clarify the exact mechanisms. The current study may provide clues for host modulation therapies targeting the prevention of tissue destruction associated with periodontal disease.

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