Journal of
PERIODONTAL RESEARCH

J Periodont Res 2012; 47: 74–80

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2011.01406.x

Preventive effects of thymoquinone in a rat periodontitis model: a morphometric and histopathological study

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Ozdemir H, Kara MI, Erciyas K, Ozer H, Ay S. Preventive effects of thymoquinone in a rat periodontitis model: a morphometric and histopathological study. J Periodont Res 2012; 47: 74–80. © 2011 John Wiley & Sons A/S

Background and Objective: Thymoquinone has a variety of pharmacologic properties, including antihistaminic, antibacterial, antihypertensive, hypoglycemic, anti-inflammatory and anti-oxidative activities. Through its anti-inflammatory and antioxidant properties, thymoquinone may play an important role in preventing periodontal diseases. The aim of this study was to evaluate the effectiveness of thymoquinone in preventing the initiation and progression of periodontitis in a rat periodontitis model.

Material and Methods: Twenty-four rats were randomly divided into three experimental groups: a nonligated (NL) treatment group ($n = 8$), a ligature-only (LO) treatment group ($n = 8$) and a ligature plus thymoquinone (10 mg/kg, daily for 11 d) (TQ) treatment group. In order to induce experimental periodontitis, a 4/0 silk suture was placed at the gingival margin of the right-mandibular first molars of the rats. Thymoquinone was administered by gastric feeding until the animals were killed on day 11. Changes in the alveolar bone levels of rats in each group were measured clinically, and tissues of rats in each group were examined histopathologically to determine inflammatory cell infiltration (ICI), osteoblast and osteoclast activities, and osteoclast morphology.

Results: Alveolar bone loss around the mandibular molar tooth was significantly higher in the LO group compared with NL and TQ groups ($p < 0.05$). The ratio of the presence of ICI and osteoclast numbers was significantly higher in the LO group than in the NL and TQ groups ($p < 0.05$). Osteoblastic activity was significantly lower in the LO group than in the NL and TQ groups ($p < 0.05$).

Conclusion: The present study showed that the oral administration of thymoquinone diminishes alveolar bone resorption in a rat periodontitis model.

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Key words: animal model; periodontal disease; periodontal therapy

Accepted for publication July 6, 2011

Periodontitis, which affects much of the world's adult population, is a destructive, inflammatory, pathological condition that affects the connective tissue attachment between the alveolar bones of the teeth. It is characterized by an excessive host response to gram-negative tooth-associated pathogens organized as a biofilm, which destroys the surrounding cells and tooth-supporting tissues, including alveolar bone, and eventually leads to tooth loss (1–3). Although oral bacteria, and toxins, enzymes and metabolites from bacteria, are considered to be

the main causative factors for the initiation of inflammatory processes, the host inflammatory response is primarily responsible for the progression of this disease and for most of the break down of dental connective tissues (4,5).

Mechanical and surgical therapy approaches, combined with antibacterial therapy, have been carried out for many years. Contemporary treatment modalities, however, which focused on exploring inflammatory pathways and mediators, as well as on new ways to control inflammation, have also been examined in detail, on a par with traditional modalities (6). Therefore, many immune-response modulatory agents, including anti-inflammatory (7,8) and antioxidant agents (9–11), were researched in the hope of preventing the deterioration of tooth-supporting tissues.

Recent data have demonstrated that increased oxidative stress and diminished antioxidant capacity within the oral cavity are two factors important in the spread of periodontal tissue destruction (12). Furthermore, patients with periodontal problems show enhanced levels of lipid peroxidase and reactive oxygen species (ROS), which cause a state of oxidative stress (13). Oxidative stress is a condition that results from a lack of equilibrium between the intracellular production of free radicals and the antioxidants that regulate oxidative reactions by inhibiting, delaying or hampering the oxidation of substances. This 'disequilibrium' between pro-oxidants and antioxidants can be disrupted by an increase in the amount of free radicals, or by a reduction in the amount of anti-oxidative substances, and can result in lipid peroxidation, DNA damage and the degradation of cellular proteins. It was demonstrated that a series of inflammatory diseases, including diabetes mellitus, atherosclerosis and chronic inflammatory lung disease, are related to oxidative stress (12). With the emerging understanding of the role of oxidative stress in periodontal disease, host-modulatory therapies using antioxidants have been investigated in detail to determine their ability to prevent or slow the break down of soft and hard periodontal tissues (9–11).

As a result of their anti-oxidative and anti-inflammatory properties, herbal therapies are also a favorite research area for the prevention and treatment of various diseases. Of these therapies, a widely used plant is *Nigella sativa*, which is used in traditional medicines in Middle-Eastern and Asian countries to treat a range of ailments, including rheumatoid pain, hypertension and asthma (14).

Thymoquinone (2-isopropyl-5-methyl-1, 4-benzoquinone), the main constituent of the volatile oil of from *N. sativa* seeds, has a range of pharmacologic properties, including antihistaminic, antibacterial, antihypertensive, hypoglycemic, anti-inflammatory and immunopotentiating actions; the antioxidant effect of thymoquinone is considered to be one of its most significant properties. It has been reported that thymoquinone induces an antioxidant response through the scavenging capability of various free radicals, with its scavenging power being as effective against superoxide anions as superoxide dismutase (15). Thymoquinone has also been argued to have anti-inflammatory potential through membrane lipid peroxidation, via eicosanoids (16).

In light of this data considering anti-inflammatory and antioxidant properties and the capability of significantly inhibiting the expression of proinflammatory cytokines, thymoquinone may be thought to play a significant role in preventing the initiation and progression of periodontitis. It is probable that it will present a new aspect in the prevention and treatment of periodontal disease.

Material and methods

The animal cohort was composed of male Wistar rats weighing 300 ± 10 g, obtained from the Experimental Animals Unit of the Faculty of Medicine from Cumhuriyet University. The animals were kept in temperature-controlled cages (approximately 25°C), exposed to a 24-h light–dark cycle of equal time, and 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.

Twenty-four rats were randomly divided into three groups: the nonligated treatment (NL) group ($n = 8$), the ligature-only (LO) group ($n = 8$) and the ligature plus thymoquinone (10 mg/kg, daily for 11 d) (TQ) group. 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 the right mandibular first molars of the rats. The sutures were checked after application, and lost or loose sutures were replaced. In the TQ group, thymoquinone was administered systemically by gastric feeding, at a rate of 10 mg/kg/d.

Daily systemic treatment with thymoquinone was continued until all rats were killed on day 11; 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

A single examiner (H. Ozdemir), who was not aware of the experimental data, carried out morphometric measurements of alveolar bone loss. After a gingival dissection was performed, the mandibles were de-fleshed and stained with 1% aqueous methylene blue solution (Merck, Rahway, NJ, USA), in order to distinguish bone from teeth. A stereomicroscope (Leica Microsystems, Wetzlar, Germany) equipped with a ×40 objective was used for measuring alveolar bone height. 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.

Histopathologic evaluation

A histologic evaluation was performed by a single examiner (H. Ozer) who was masked to the identity of the samples. The specimens were fixed in a

10% neutral-buffered formalin solution and demineralized in an aqueous 10% formic acid solution. The 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.* 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 interdental 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 semiquantitative scoring as not visible ICI (score = 0), slightly visible ICI (score = 1) and dense ICI (score = 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 the score was 0, mild to moderate visibility was scored as 1 and existing osteoblastic activity with a dense mass was scored as 2.

Statistical analysis

The ratios of the presence of ICI and osteoblastic activity were analyzed using a chi-square test. Osteoclast numbers and alveolar bone loss were analyzed using an analysis of variance test, followed by a Tukey test for pairwise comparisons. Data were presented as mean ± standard deviation or as a percentage, as appropriate. $p < 0.05$ was considered statistically significant.

Results

The animals did not show any obvious signs of systemic illness throughout the study period. Weight loss was < 10% in the LO and TQ groups on day 11.

Measurements of alveolar bone loss in mandibular molars revealed significantly higher bone-loss values in the LO group compared with NL and TQ groups ($p < 0.05$) (Figs 1 and 2A–C). The alveolar bone loss in the NL group was less than in the TQ group, but the difference was not significant between the two groups ($p > 0.05$).

Figure 3 presents the number of osteoclasts in the study groups. Osteoclasts were found in all rats, except for one in the NL group. Significantly higher numbers of osteoclasts were found in the LO group compared with the NL and TQ groups ($p < 0.05$). The osteoclasts had ruffled borders in the NL group. The percentage of osteoclast morphologies assigned a score of 2 in the TQ group was higher than in the NL and LO groups (87.5% vs. 12.5% and 0%, respectively; $p < 0.05$) (Fig. 4A).

The ratio of the presence of ICI in the LO group was significantly higher than those of the NL and TQ groups (87.5% vs. 12.5% and 37.5%) ($p < 0.05$) (Figs 4B and 5).

In the LO group, osteoblastic activity was significantly lower than in the NL and TQ groups (25% vs. 87.5% and 100%, respectively; $p < 0.05$).

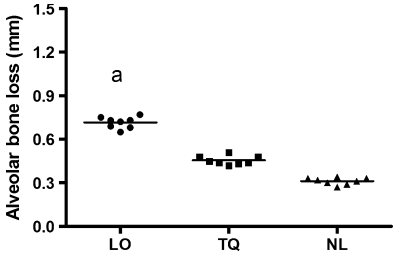

Fig. 1. Mean alveolar bone loss in the nonligated (NL), ligature-only (LO) and ligature plus thymoquinone (TQ) groups. $p < 0.05$ vs. LO, TQ and NL groups. Lines = mean values.

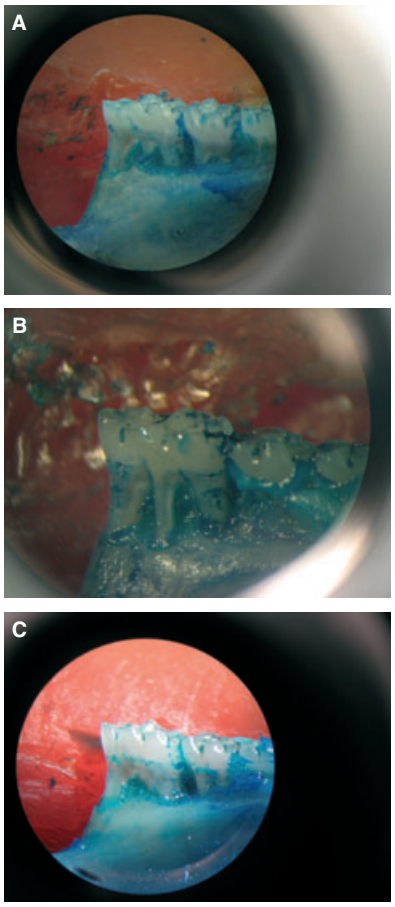

Fig. 2. (A) Representative image of alveolar bone loss in mandibular first molars in the nonligated (NL) group. (B) Image of alveolar bone loss in mandibular first molars in the ligature-only (LO) group. (C) Image of alveolar bone loss in mandibular first molars in the ligature plus thymoquinone (TQ) group.

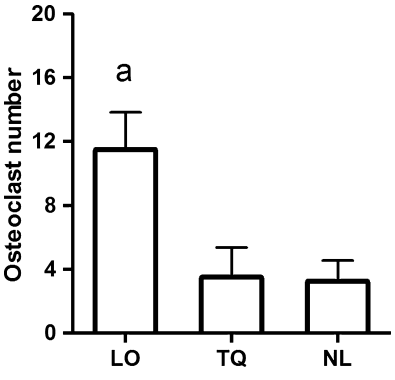

Fig. 3. Osteoclast numbers in the nonligated (NL), ligature-only (LO) and ligature plus thymoquinone (TQ) groups. $p < 0.05$ vs. the LO, TQ and NL groups. Lines = mean values.

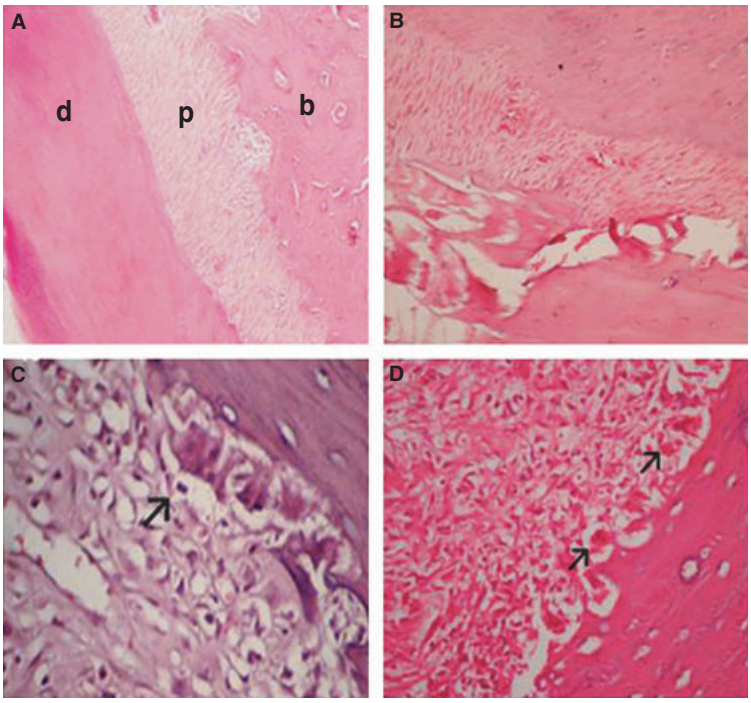

Fig. 4. Histopathology of mandibular first molar tooth in all groups. (A) Normal mandibula, showing alveolar bone (b), periodontal ligament (p), and dentin (d) (hematoxylin & eosin stain; original magnification ×25). (B) Mandibular histology after 11 d of periodontitis in the ligature-only (LO) group, showing severe inflammatory cell infiltration (ICI) in the periodontal ligament (hematoxylin & eosin stain; original magnification ×50). (C) Mandibula in the LO group after 11 d of periodontitis, showing mainly alveolar bone with numerous osteoclasts with ruffled borders (arrow) (hematoxylin & eosin stain; original magnification ×100). (D) Mandibula in the ligature plus thymoquinone (TQ) group after 11 d of periodontitis, showing increased osteoblastic activity (arrows) (hematoxylin & eosin stain, original magnification ×100).

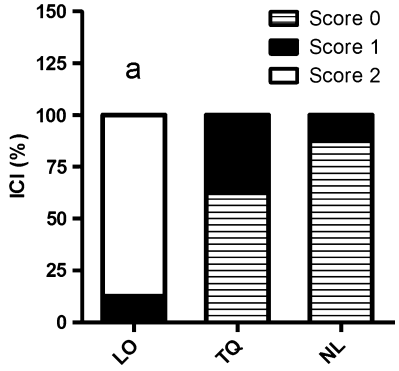

Fig. 5. Frequency (%) of inflammatory cell infiltration (ICI) in the groups. LO, ligature only; NL, nonligated; TQ, ligature plus thymoquinone.

Discussion

Periodontitis is a complicated disorder dependent on a number of factors, but inflammatory cell accumulation induced by microorganisms in the gingival connective tissues is considered to be the main causative factor. The majority of bacteria are located in the periodontal pocket and do not invade the periodontal tissues; therefore, these microorganisms can never be removed effectively by the host immune system. Consequently, chronic inflammation and extreme, unceasing host responses, including the recruitment of leukocytes and the subsequent release of inflammatory mediators and cytokines, can cause periodontal pathology (17,18). Bacterial products in the immune cells, such as lipopolysaccharides, promote local factor production, including interleukin (IL)-1α, IL-1β, prostaglandin E2 and tumor necrosis factor-alpha (TNF-α). IL-1α, IL-6 and TNF-α, like proinflammatory cytokines, can also induce differentiation of osteoclast precursors and osteoclast activity, which leads to bone resorption (11). Although these factors were considered to be the main causative factors, the onset and progression of periodontal disease is dependent on a number of host risk factors, such as age, sex, race and smoking status (17). Lastly, the role of the oxidants–antioxidants in periodontitis has been investigated in detail by the researchers (19).

By the time the disequilibrium between the production of ROS and tissue concentration of antioxidants in favor of ROS oxidative stress rises, which can lead to endothelial cell damage and increased microvascular permeability, the formation of chemotactic factors, such as leukotriene B4, the recruitment of neutrophils at inflammation sites, lipid peroxidation and oxidation, DNA single-strand damage and the formation of peroxynitrites, has occurred (4,20). The areas of bone resorption, ROS and especially superoxide play a role as intermediate molecules in the activation of osteoclasts (21).

Antioxidants are present in many foodstuffs that hinder oxidative processes and may play a vital role in preventing numerous degenerative and chronic diseases, such as cardiac disease, brain dysfunction, immune system decline and cancer (9,13,22,23). It was shown that antioxidants, such as ascorbic acid, α-tocopherol, glutathione peroxidase and superoxide dismutase, help to balance excessive ROS formation by delaying or hindering the oxidation of substrates (24). Considering excessive oxidant release and antioxidant deficiency supporting periodontal tissue breakdown, antioxidants such as calcium gluconate (7), propolis (9) and curcumin (25) have been tested as a result of their anti-inflammatory and antioxidant properties.

Ku *et al.* (7) evaluated the effects of calcium gluconate on ligature-induced experimental periodontitis and suggested that daily oral treatments with calcium gluconate effectively inhibit periodontitis and related alveolar bone loss via antioxidant effects.

Toker *et al.* (9) examined the morphometric and histopathologic

changes associated with experimental periodontitis in rats responding to propolis, and demonstrated that the systemic administration of propolis prevents alveolar bone loss.

As a result of its rich and diverse chemical composition, *N. sativa* and its main constituent, thymoquinone, have been investigated in detail. *N. sativa* has long been used in folk medicine to promote good health and to treat disease. According to recent studies, it was suggested that extracts of *N. sativa*, thymoquinone in particular, have many therapeutic effects, including antioxidant, anti-inflammatory, antimicrobial, anti-tumor, immunomodulatory, bronchodilatory, hypotensive, antidiabetic, hepatoprotective, gastroprotective, antihistaminic and neuroprotective (14–16,26).

As a result of its antioxidant and anti-inflammatory properties, thymoquinone has cytoprotective effects (14). Tekeoglu *et al.* (16) aimed to examine the anti-inflammatory effects of thymoquinone on arthritis in rat models, and reported that thymoquinone suppressed adjuvant-induced arthritis in rats. They also stated that TNF-α and IL-1β levels in the thymoquinone-treated group were significantly lower than in the control group. Houghton *et al.* (27) explored the mechanisms of the anti-inflammatory effects of thymoquinone and stated that thymoquinone inhibited thromboxane and leukotriene B4 synthesis from eicosanoids by inhibiting the cyclooxygenase and lipooxygenase enzymes. Vaillancourt *et al.* (28) aimed to evaluate the *in vitro* and *in vivo* effects of thymoquinone on rheumatoid arthritis and demonstrated that thymoquinone was effective in decreasing the arthritis score, osteoclastogenic activity and bone resorption. The results of our study were in accordance with another study (16,27,28), in which thymoquinone decreased ICI and osteoclastic activity. This study also demonstrated that thymoquinone breaks down the osteoclast morphology.

The strong antioxidant potential of thymoquinone was related to the scavenging ability of different free radicals. It can inhibit the cyclooxygenase and 5-lipooxygenase pathways of arachidonic acid metabolism, as well as membrane lipid peroxidation (15,29). It was also indicated that thymoquinone showed protective action against the damaging effects of superoxide anion radicals. In addition, the increased glutathione and superoxide dismutase levels produced a marked inhibition in the release of leukotrienes by thymoquinone, adding further support for its protective action (30).

Because of numerous studies (14,16,29,30) reporting that thymoquinone inhibits tissue inflammation and oxidative stress, we thought that thymoquinone may play a role in preventing the onset and progression of periodontitis. Therefore, we aimed to explore the effects of oral administration of thymoquinone on periodontitis, in an experimental rat model.

Because of the limitations on researching periodontal disease symptoms and treatments in humans, animal studies are most commonly used by researchers (4). Molars in rats are similar in anatomic configuration and structure to human molars, but are smaller, so it was difficult to perform any sort of periodontal treatment on these teeth (31). A further limitation to the experimental model used is that the induced periodontitis follows an acute course, during which tissue trauma and adjacent accumulation of microbes accelerate the destructive process. Such pathways of acute inflammation are likely to differ from chronic periodontitis (4). There are, however, inherent limitations to animal models of human disease. Rats, mice, dogs and primate-like animals have been frequently preferred for periodontal research. Considering cost, size and maintenance requirements, rats were used in this study.

The serum levels of certain agents and serum metabolites, for example, prooxidant–antioxidant levels, reactive oxygen metabolites and TNF-α, were analyzed in a detailed investigation of antioxidant agents in a periodontitis study (10). In the present study we only evaluated changes in alveolar bone levels clinically and histopathologically. Hence, further studies are needed to evaluate serum levels of thymoquinone and other serum metabolites.

For assessment of alveolar bone level, radiographic and clinical periodontal parameters such as deep pockets, plaque index and clinical attachment loss can be evaluated (32). Because the principal aim of this study was to evaluate alveolar bone loss, we did not need to evaluate other periodontal parameters. In this study, stereomicroscopy, rather than radiography, was preferred for evaluating alveolar bone loss.

In the available literature, various techniques have been used for inducing periodontitis in rats, such as the introduction of pathogenic microorganisms, dietary manipulation and the placement of ligatures (33–35). As placing a ligature around the cervix of the right mandibular first molar in rats is predictable and favored by researchers, we opted for this technique. Placing a ligature caused mechanical trauma and bacterial retention, resulting in periodontal tissue detachment and an intense host–plaque interaction (9). According to our results, ligature placement on the first molar tooth caused a significant amount of bone loss.

Lima *et al.* (36) demonstrated that placement of a ligature around the molar teeth of rats leads to the appearance of inflammatory cells, including osteoclasts and lymphocytes, beneath the ligature. They also stated that considerable alveolar bone loss commenced on day 3 of periodontitis induction, reached a maximum between days 7 and 11, and declined by day 14. These data support the results of Sallay *et al.* (37), who suggested that maximum bone loss was achieved by day 9 after ligature placement. In our study, the ligature was placed around the first molar teeth for 11 d, as in Toker *et al.* (11).

In the present study, alveolar bone loss was measured in order to determine the distance between the alveolar bone crest and the cemento–enamel junction. This is a highly accepted and preferred method for researchers. The results of this study revealed that more bone was lost in the LO group than in TQ and NL groups. No statistically significant difference was found in alveolar bone loss between the NL and

TQ groups. It was also revealed that osteoclastic activity and ICI was higher in the LO group than in the other groups. No difference was seen in ICI and osteoclast number between the NL and TQ groups. These morphological and histological results showed that systemic administration of 10 mg/kg of thymoquinone diminishes periodontal inflammation and alveolar bone loss in experimental periodontitis.

A different dose of thymoquinone have been used by researchers. For evaluating anti-inflammatory, anticancer, antioxidant and cytoprotective effects, the most commonly used dose of thymoquinone ranged from 5 to 12.5 mg/kg, at which no significant adverse effects were observed (38). According to some studies, no adverse reaction has been encountered, except in children receiving high doses of 80 mg/kg of thymoquinone (30). Al-Ali *et al*. (38) reported that the 50% lethal dose (LD_{50}) levels of orally administered thymoquinone were 10 times higher than the LD_{50} levels of intraperitoneally administered thymoquinone, in both mice and rats. It was also suggested that thymoquinone was a relatively safe compound in experimental animals, especially when given orally. In this study, 10 mg/kg of thymoquinone was used, daily for 11 d.

Conclusion

Oral administration of thymoquinone prevented periodontal inflammation and decreased alveolar bone loss in experimental periodontitis in a ligature-induced rat model. Considering its potent antioxidant and anti-inflammatory properties and minimal adverse effects, thymoquinone may provide additional new insights into treatment schemes for preventing periodontitis.

Acknowledgments

The authors thank Associate Professor Hülya Toker, Cumhuriyet University School of Dentistry, Department of Periodontology, Sivas, Turkey, for editorial assistance with the final preparation of the manuscript.

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