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The effect of local application of thymoquinone, *Nigella sativa*'s bioactive component, on bone healing in experimental bone defects infected with *Porphyromonas gingivalis*

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ABSTRACT

Purpose: This study was performed to evaluate the influence of local application of thymoquinone (TQ) on bone healing in experimental bone defects infected with *Porphyromonas gingivalis* (PG).

Methods: Forty-two female rats were randomly divided into 6 groups. A bone defect was created on the right tibia of all animals. The PG, PG/collagen membrane (COL) and PG/TQ/COL groups were infected with PG. In the COL and PG/COL groups, the defects were covered with a COL; in the TQ/COL and PG/TQ/COL groups, the defects were covered with a TQ-containing COL. After 28 days, all animals were sacrificed. Quantitative measurements of new bone formation and osteoblast lining, as well as semiquantitative measurements of capillary density and tissue response, were analyzed. Furthermore, the presence of bacterial infections in defect areas was evaluated.

Results: The new bone formation, osteoblast number, and capillary density were significantly higher in the TQ groups than in the control groups ($P<0.001$, $P<0.001$, and $P<0.01$, respectively). In a comparison between the TQ/COL group, with a TQ-containing COL (TQ/COL), and the PG-infected TQ-containing COL (PG/TQ/COL) group, the newly formed bone and capillary density were higher in the TQ/COL group ($P<0.01$). When the control group was compared to the PG, PG/COL, and PG/TQ/COL groups in terms of tissue response, the differences were statistically significant ($P<0.001$, $P=0.02$, and $P=0.041$, respectively). The intensity of the inflammatory cell reaction was higher in the PG, PG/COL, and PG/TQ/COL groups ($P<0.05$).

Author Contributions

Conceptualization: Ayşe Yılmaz Baştuğ, Ceyda Özçakır Tomruk, Elif Güzel, İlkay Özdemir, Gonca Duygu, Esmâ Kütan, Gül Merve Yalçın Ülker, Fatma Özen Arıcı ; Formal analysis: Ayşe Yılmaz Baştuğ, Elif Güzel, İlkay Özdemir, Gül Merve Yalçın Ülker; Investigation: Ayşe Yılmaz Baştuğ, Elif Güzel, İlkay Özdemir, Gül Merve Yalçın Ülker; Methodology: Ceyda Özçakır Tomruk; Resources: Ayşe Yılmaz Baştuğ, Esmâ Kütan; Supervision: Ceyda Özçakır Tomruk; Writing - original draft: Ayşe Yılmaz Baştuğ, Elif Güzel, İlkay Özdemir, Gonca Duygu, Esmâ Kütan, Esmâ Kütan, Gül Merve Yalçın Ülker; Writing - review & editing: Ceyda Özçakır Tomruk, Gonca Duygu, Fatma Özen Arıcı.

Conflict of Interest

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

Conclusions: Within the limitations of this study, the local application of a TQ-containing COL positively affected bone healing even if the bone defects were infected. The results suggest that TQ increased angiogenesis and showed promise for accelerating bone defect healing. Further research is warranted to support these findings and reach more definitive conclusions.

Keywords: Bone; *Nigella sativa*; *Porphyromonas gingivalis*; Rats; Thymoquinone; Tibia

INTRODUCTION

In the oral and maxillofacial region, to promote the healing of congenital and/or acquired osseous deformities, several guided bone regeneration (GBR) techniques have been described, which involve the application of various types of bone grafts, combinations thereof, and barrier membranes or bone substitute materials [1]. The size and configuration of the bone defect are factors that influence the selection of materials. Some investigators have proposed classifications and recommendations for GBR techniques. GBR is a reliable methodology for the augmentation of horizontal defects using both resorbable and non-resorbable membranes in the majority of cases [2]. GBR techniques use barrier membranes to prevent the proliferation of soft tissue cells in order to protect blood clots and created defects [3]. Although there are many types of membranes for this purpose, collagen-originated bioresorbable barrier membranes are frequently applied in GBR techniques. Although very successful results have been reported with the use of non-resorbable membranes, some authors have indicated that these types of membranes may be more prone to complications [4]. If barrier membranes are not adequately stabilized in the application area, they can migrate to the wound edges and cause them to open. In such a case, the barrier membrane in contact with the oral flora may be infected by bacteria, disrupting wound healing [5]. Some authors have reported that preventing bacterial colonization is a key factor for the regeneration of new bone tissue [6].

Among the major periodontal pathogens, *Porphyromonas gingivalis* (PG) appears to be one of the prime etiological agents in the pathogenesis and progression of the inflammatory events of periodontal disease [7]. The induction and progression of periodontal tissue destruction are complex processes involving plaque accumulation, the release of bacterial substances, and the host inflammatory response. PG is known to produce a repertoire of virulence factors that could penetrate the gingivae and cause tissue destruction directly or indirectly, by inducing inflammation [7]. With the high frequency of PG in adult periodontitis lesions, it is strongly hypothesized that PG interacts with other members of the host microbiota by synthesizing various pathogenic factors, leading to disease progression. PG infection models have been widely used to study immune-mediated periodontal bone resorption [8,9]. Han et al. [10] suggested that PG infection-associated periodontal bone resorption is receptor activator of nuclear factor kappa-B ligand (RANKL)-dependent and is accompanied by increased local infiltration of RANKL-expressing T and B cells. Kütan et al. [11] used PG in rat tibia defects to achieve effective infection.

Although there are empirical data in the literature supporting the successful use of GBR techniques in bone regeneration, some challenging clinical situations and complications still exist [12]. To accelerate the GBR outcomes, supplementary regenerative approaches have been investigated. Recent advances in biomaterial design, drug delivery, and biologic

agents offer less invasive technologies to accelerate tissue repair and regeneration in a more predictable manner [13]. However, the aforementioned treatment modalities have not yet been implemented in clinical practice due to some limitations. These challenging factors have prompted researchers to conduct experiments on alternative treatments for future use. As alternative treatment methods used for traditional or current medical applications, herbal therapies have been claimed to have therapeutic properties, but these effects have not yet been proven by scientific methods. A medicinal herb used for herbal treatment is *Nigella sativa* (NS). A significant number of studies have been conducted on NS, and many of its pharmacological properties have been investigated, including antidiabetic, anticancer, immunomodulatory, analgesic, antimicrobial, anti-inflammatory, spasmolytic, bronchodilator, hepatoprotective, renoprotective, gastroprotective, and antioxidant actions [14,15]. A search of the literature reveals that NS can be suggested as a complementary medication or a dietary supplement for individuals with metabolic diseases due to its preventive and relieving actions [16]. The major therapeutic factor of this plant is thymoquinone (TQ), which constitutes the main component of its essential oil. The safety of TQ has been confirmed in toxicological studies, specifically when administered orally to experimental animals [17]. It is known that TQ reduces the levels of interleukin (IL)-1 β , IL-6, and tumor necrosis factor-alpha, which are known to be proinflammatory cytokines, by affecting free radicals produced by oxidative stress and thereby preventing bone resorption [18]. In addition, it has been shown that TQ accelerates bone healing through its antioxidant properties [19]. Kara et al. [15] reported that the systemic application of TQ in maxillary palatal expansion increased bone healing. Ozdemir et al. [20] showed that systemic TQ application in a rat periodontitis model prevented alveolar bone resorption. Although studies have investigated the effects of systemically applied TQ on bone [15,18,20,21], no studies have evaluated the local effects of TQ to the best of the authors' knowledge. Therefore, this study aimed to investigate the antimicrobial and bone healing-enhancing effects of a TQ-releasing collagen membrane (COL) on both infected and noninfected defects in rat tibias.

MATERIALS AND METHODS

The study was approved by the local ethics committee of Yeditepe University (395/2014 YÜDHEK, Istanbul, Turkey). The animal cohort used in this study comprised 3-month-old Sprague Dawley female rats (200–250 g). Standard animal care guidelines were applied throughout the entire study. Forty-two rats were randomly divided into 6 groups (**Table 1**):

In all experimental groups, a critical-sized defect (CSD) was created on the tibia.

Group I (control): served as control and was left to heal without intervention.

Group II (PG): infected with PG.

Group III (COL): not infected with PG, and a COL was applied.

Table 1. Description of the groups

Group name	Number	PG	Application of COL	Application of TQ-releasing COL
Control	7	-	-	-
PG	7	Infected	-	-
COL	7	-	Yes	-
PG/COL	7	Infected	Yes	-
TQ/COL	7	-	-	Yes
PG/TQ/COL	7	Infected	-	Yes

COL: collagen membrane, PG: *P. gingivalis*, TQ: thymoquinone.

Group IV (PG/COL): infected with PG, and a COL was applied.

Groups V (TQ/COL): not infected with PG, and a TQ-containing COL was applied.

Group VI (PG/TQ/COL): infected with PG, and a TQ-containing COL was applied.

Surgical procedure

Before surgery, the rats were anesthetized using a combination of ketamine (20 mg/kg) and xylazine (10 mg/kg). The right side of all rat tibias was then shaved and disinfected. The same operative techniques were applied for flap elevation. A CSD with a diameter of 3×3 mm² previously described by Ribeiro et al. [22] was created on the medial cortex of each tibia with a trephine bur (**Figure 1A**) in all groups.

The defects of the rats in the PG, PG/COL, and PG/TQ/COL groups were infected with 100 μL of the prepared solution containing 10⁹ colony forming unit (CFU)/mL PG in phosphate-buffered saline (PBS) (optical density approximately 0.8 at 660 nm) (**Figure 1B**) [11].

After infection, a local COL of bovine origin containing 2.6% type 1 fibrillar collagen gel (5 mg collagen in a single 0.2-mm layer) (Coll, The National Research and Development Institute for Textiles and Leather, Bucharest, Romania) [23] was applied to the COL and PG/COL groups (**Figure 1C**).

In the TQ/COL and PG/TQ/COL groups, a TQ-containing (2%) COL from the same institution was applied (**Figure 1D**). The incision was closed with simple sutures subcutaneously and superficially.

Bacterial culture preparation

PG ATCC 3744 (ATCC, Rockville, MD, USA) was cultivated on brain heart infusion agar containing 5% sheep blood (Salubris, Gaithersburg, MD, USA) under aseptic conditions and incubated for 72 hours at 37°C under anaerobic conditions (10% H₂, 10% CO₂, and 80% N₂ gas mixture, Habas, Soğanlık/Kartal, Turkey) [11]. Next, the rats were anaesthetized and inoculated with 100 μL of 10⁹ CFU/ml PG (optical density of up to 0.8 at 660 nm) in PBS (Gibco, Invitrogen, Waltham, MA, USA).

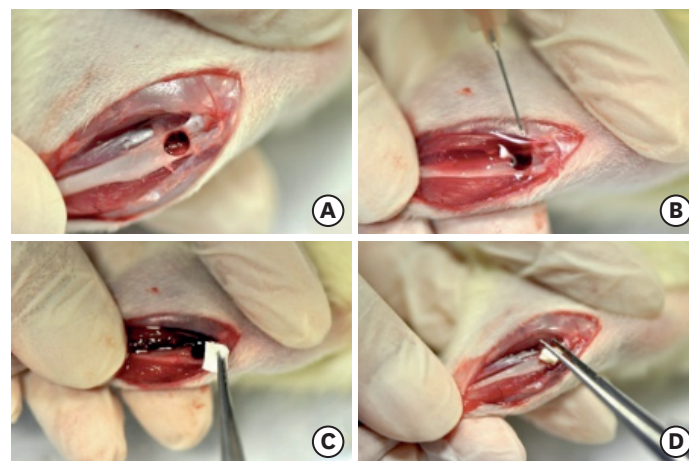


Figure 1. Surgical procedures of the study. (A) A 3×3 mm² defect, (B) microbial infection, (C) collagen covering, (D) coverage with collagen containing thymoquinone.

TQ-containing COL preparation

The TQ oil was extracted from NS plants collected in the province of Hatay. The plants were dried at around 20°C–22°C in a dark place at the Laboratory of Medicinal and Aromatic Plants, Department of Field Crops, Faculty of Agriculture, Mustafa Kemal University. TQ essential oil (0.2%) was obtained from dry leaves by steam distillation in a neo-Clevenger apparatus. The concentration of each collagen gel was adjusted at 1.2% and a pH of 7.4 with 1 M sodium hydroxide (under stirring). Using the patented method of encapsulation of essential oil in collagen, the TQ oil did not evaporate or melt during processing [23,24]. Next, 0.2% TQ oil was incorporated into 100 mL of collagen gel. By freeze-drying, the oil was kept in the pores of the COL. Each 1-cm square of COL included 0.01 mL of TQ oil.

Twenty-eight days after surgery, all animals were sacrificed on the same day with an overdose of an anesthetic injection. During dissection for bone harvesting, the soft and hard tissues were assessed clinically for pus and/or abscess formation. One of the tissue samples in each group (n=6) was set aside for a microbiological evaluation and the other tissue samples (n=36) were placed into 10% neutral buffered formalin (pH 7.0) for histopathological and histomorphometric evaluations.

Histopathological and histomorphometric evaluations

Bone tissue specimens were removed and fixed with 10% neutral buffered formalin (pH 7.0, 20°C–22°C). After decalcification in De Castro solution (chloral hydrate, nitric acid, distilled water), all samples were dehydrated in a graded series of ethanol for embedding in paraffin using an automated tissue processor (Microm International GmbH part of Thermo Fisher Scientific Spin Tissue Processor, STP-120, Microm International GmbH, Walldorf, Germany). Five-micrometer-thick sections were cut with a sliding microtome (Microm International GmbH part of Thermo Fisher Scientific, HM 430, Microm International GmbH). All sections were stained with hematoxylin and eosin (HE) and Masson trichrome (MT) to evaluate newly-formed bone, the osteoblast lining, capillary density, and tissue response. Two independent investigators evaluated the bone defect area using a light microscope (BX61, Olympus, Tokyo, Japan) attached with a computerized digital camera (DP72, Olympus). In this study, a histomorphometric analysis was performed to evaluate the newly formed bone and osteoblast count on the bone surface (osteoblast lining). The capillary density and tissue response were evaluated with a semi-quantitative scoring system.

Quantitative analysis of newly formed bone and osteoblast lining

Analyses of bright-field images were performed quantitatively with an image-processing program (DP2-BSW, Olympus). The number of pixels in each image corresponding to the new trabecular bone area (μm^2) was quantified and divided by the total number of pixels corresponding to the total defect area (μm^2); in this way, a percentage (%) for each sample was obtained (DP2-BSW, Olympus).

Osteoblasts were quantified based on their morphology on HE-stained sections for the length of their linear apposition along the new bone surfaces relative to the total new bone surface length in 3 randomly selected high-power fields ($\times 200$). These values were reported as a percentage (%) for each sample [25].

Semi-quantitative analysis of capillary density and tissue response

Semi-quantitative analyses of each sample were scored by 2 different investigators, who were blinded and calibrated with each other. The capillary density of the connective tissue

surrounding the bone trabeculae was rated as mild (+: 0–15 cells), moderate (+: 16–30 cells) or strong (+++: >30 cells) [15]. The tissue response was analyzed based on the presence of fibrotic connective tissue formation and inflammatory cell infiltration. Inflammatory cell infiltration was rated using a score between 0 and 4 [26].

Microbiological evaluation

Defect areas (n=6) infected with PG were cut sagittally, and bone samples were weighed and then ground in 3 mL of PBS. The suspension was diluted (1:1, 1:10, 1:100, and 1:1,000) and 100 µL of each dilution was seeded on 5% sheep blood-brain, heart infusion medium and incubated (37°C, 72 hours, anaerobic conditions). The infection was confirmed when culture growth was determined as positive on agar plates. Dividing the total number of CFUs per gram by the total initial weight of the bone sample gave the number of CFUs per gram of bone for each dilution. When the average of all dilutions was calculated, the number of CFUs per gram of bone for each animal was determined.

Statistical analysis

In the analysis of new bone area, the osteoblast lining, and the number of capillaries, the Shapiro-Wilk test showed a normal distribution of data. Therefore, these parameters were analyzed with 1-way analysis of variance, followed by *post hoc* Holm-Sidak testing. Statistical calculations were performed using Sigma plot for Windows, version 12.0 (Systat Software Inc., San Jose, CA, USA). A *P*value < 0.05 was considered to indicate statistical significance.

RESULTS

Clinical findings

None of the animals were lost during the surgical procedure or in the postoperative period. During the sacrifice process, clinical findings of infection (pus formation) were observed in the tibias of the rats in the PG group (**Figure 2**), while there were no similar signs of infection in the other groups.

Histopathologic and histomorphometric evaluations

Descriptive statistical data from the present study are given in **Table 2**. Photomicrographs of HE- and MT-stained tissue sections from all groups are presented in **Figure 3**.

The histologic evaluation showed that the ossification process, which was initiated in the cortical bone, progressed towards the center of the defect. There was a high number of



Figure 2. Observation of pus formation in the *P. gingivalis* group.

Table 2. New bone area, osteoblast number, capillary density, and tissue response in all groups

Parameter	Group	Mean	SD	SE
Bone repair	Control	0.200	0.0632	0.0258
	PG	0.167	0.0816	0.0333
	COL	0.233	0.0516	0.0211
	PG/COL	0.233	0.121	0.0494
	TQ/COL	0.775 ^{a)}	0.0758	0.0310
	PG/TQ/COL	0.500 ^{a)}	0.237	0.0966
Osteoblast lining ratio	Control	0.345	0.0404	0.0165
	PG	0.313	0.0497	0.0203
	COL	0.350	0.0498	0.0203
	PG/COL	0.328	0.0331	0.0135
	TQ/COL	0.675 ^{a)}	0.0666	0.0272
	PG/TQ/COL	0.597 ^{a)}	0.0698	0.0285
Capillary density	Control	0.667	0.816	0.333
	PG	0.500	0.548	0.224
	COL	0.667	0.816	0.333
	PG/COL	0.500	0.837	0.342
	TQ/COL	2.500 ^{a)}	0.837	0.342
	PG/TQ/COL	2.333 ^{a)}	0.816	0.333
Response rate	Control	0.667	0.516	0.211
	PG	3.167 ^{a)}	0.753	0.307
	COL	0.500	0.548	0.224
	PG/COL	2.000 ^{a)}	0.894	0.365
	TQ/COL	0.500	0.548	0.224
	PG/TQ/COL	1.833 ^{a)}	0.753	0.307

SD: standard deviation, SE: standard error, COL: collagen membrane, PG: *P. gingivalis*, TQ: thymoquinone.

^{a)}P<0.05.

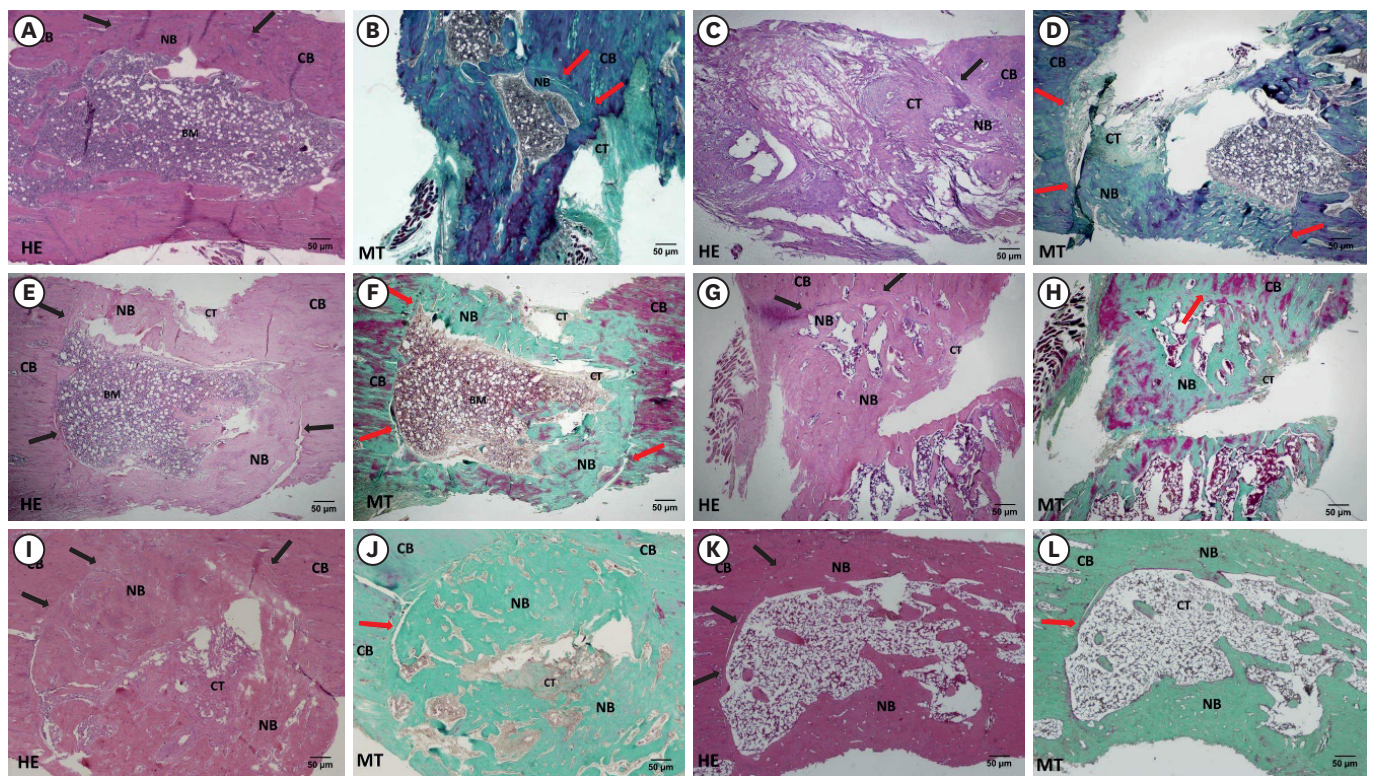


Figure 3. Histological view of the defect area in each group obtained on day 28 (A, B: control, C, D: PG, E, F: COL, G, H: PG/COL, I, J: TQ/COL, K, L: PG/TQ/COL). A fibrous callus and a small number of new bone trabeculae filled the defect area in the control group. The defect area was almost filled with fibrous callus in the PG group. The number of new bone trabeculae in the COL and PG/COL groups were similar. There was a higher number of new bone trabeculae only in the TQ groups (TQ/COL and PG/TQ/COL) compared to others. CB: cortical bone, NB: new bone, CT: connective tissue, BM: bone marrow, arrows: defect border, HE: hematoxylin and eosin, MT: Masson trichrome, COL: collagen membrane, PG: *P. gingivalis*, TQ: thymoquinone.

active osteoblasts that produced a new bone matrix at the junction of the new trabecular bone. Furthermore, the marrow space was occupied by hematopoietic precursor cells and fat islands (Figure 3).

Quantitative analysis of newly formed bone tissue and osteoblast lining

When the ratio of the newly formed bone area to the total bone defect area was evaluated; there were no statistically significant differences between the control group and the PG, COL, and PG/COL groups ($P>0.05$). The TQ/COL and PG/TQ/COL groups showed significantly higher new bone ratios than other groups ($P<0.001$). When the TQ/COL and PG/TQ/COL groups were compared, the newly formed bone ratio was significantly lower in the PG/TQ/COL group ($P<0.01$) (Figure 4A).

Although no statistically significant difference was observed between the control group and the PG, COL, and PG/COL groups ($P>0.05$), a statistically significant difference was observed ($P<0.001$) in the TQ groups in terms of the osteoblast lining on the new bone surface. The number of osteoblasts was statistically significantly higher in the TQ groups than in the other groups ($P<0.001$). No significant difference in this regard was found between the TQ/COL and PG/TQ/COL groups ($P>0.05$) (Figure 4B).

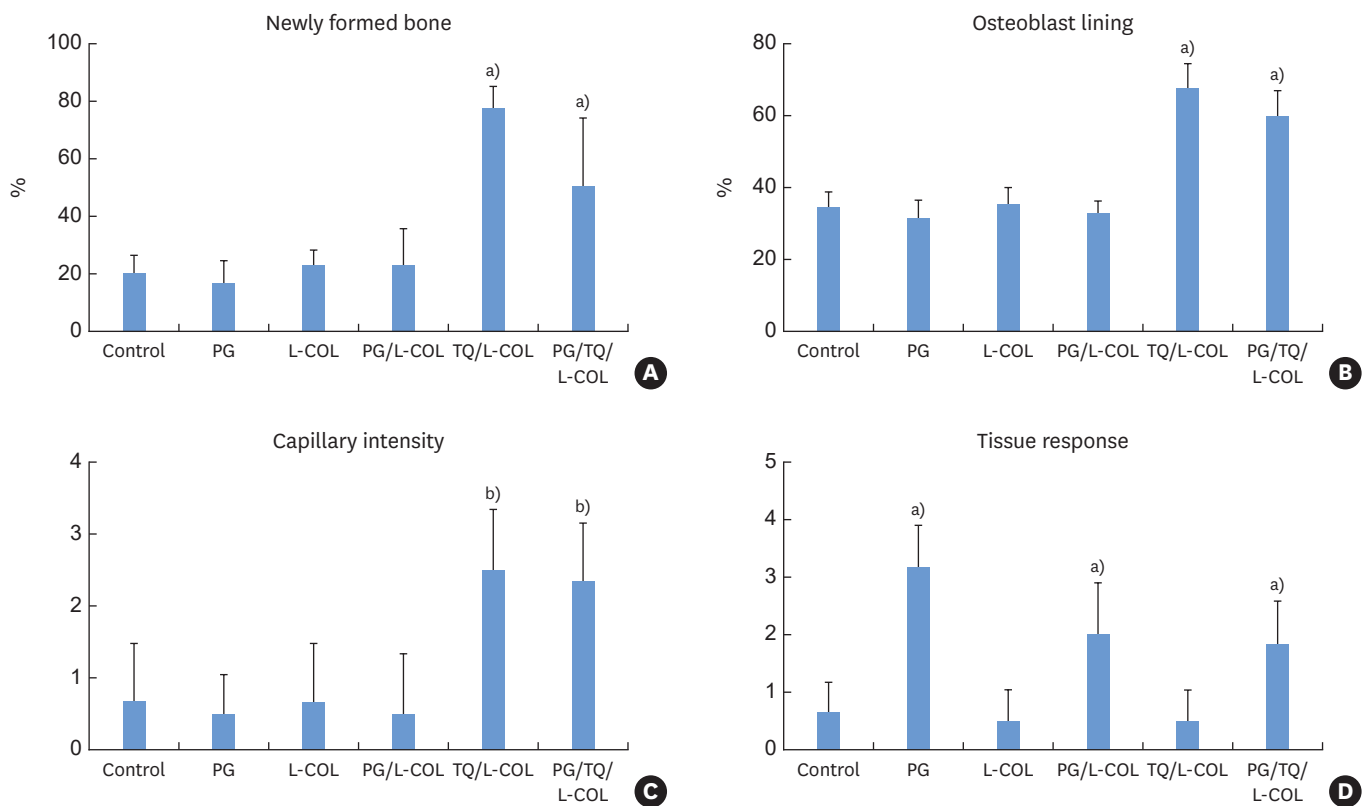


Figure 4. Histological and histomorphometric effects of TQ on defect healing. (A) Effect of TQ on newly formed bone. (B) Effect of TQ on osteoblast lining. (C) Effect of TQ on capillary density. (D) Effect of TQ on tissue response.

^{a)}Statistically significant values are indicated with $P<0.001$; ^{b)}Statistically significant values are indicated with $P<0.01$.

COL: collagen membrane, PG: *P. gingivalis*, TQ: thymoquinone.

Semi-quantitative analysis of capillary density and tissue response

No statistically significant difference in capillary density was found between the control group and the PG, COL, and PG/COL groups ($P=1$). The capillary density was significantly higher in the TQ/COL and PG/TQ/COL groups than in the other groups ($P<0.01$). Capillary density was higher in the TQ/COL group than in the PG/TQ/COL group, but the difference was not statistically significant ($P>0.05$) (Figure 4C).

When all groups were compared, the intensity of the inflammatory cell reaction was higher in the PG, PG/COL, and PG/TQ/COL ($P<0.05$). When the PG-infected groups were compared with their corresponding controls (the control, COL, and TQ/COL groups), a significant difference was observed ($P<0.001$). In the PG groups, moderate to severe inflammatory activity was noted, with the infiltration of macrophages, lymphocytes, fibroblasts, and some foreign-body giant cell polymorphonuclear leukocytes (Figure 3C and D).

No statistically significant difference was found between the COL and TQ/COL groups (Figure 4D).

Microbiological findings

Microbiologically, 10×10^4 CFUs/g of bacteria were observed only in the PG group. No bacterial growth was observed in the other groups.

DISCUSSION

In this study, the effect of a TQ-containing COL applied locally on bone defects in the rat tibia on bone healing was evaluated. Furthermore, defects were infected with bacteria in order to mimic the complication of membrane infection, which is one of the most serious problems encountered in the treatment of oral bone defects. Bone healing in the defect area was evaluated microbiologically, histologically, and histomorphometrically.

Severe bony defects due to tooth loss often result in deterioration of the original ridge dimensions. GBR has been shown to be effective for restoring bone volume to improve functional and aesthetic outcomes in patients. Bioactive membranes derived from collagen are a popular choice in tissue engineering applications [1,2]. To improve the properties of COLs, several materials have been added, such as doxycycline [11]. A clinical study showed that periodontal chips containing TQ showed significant improvements in clinical parameters when used as an adjunctive therapy to treat chronic periodontitis [27].

The CSD model, which was developed by Hollinger and colleagues, is considered the gold standard in bone tissue engineering applications. In the CSD model, it is accepted that the bone defect itself will not recover during the life of the animal without an external application [28]. There are many studies in the literature on rat tibia related to bone regeneration and many different opinions regarding the defect size. The exact diameter of CSDs and non-CSDs is still being debated. While some researchers create 2×2 mm defects in the tibia of rats [29], many other studies have created a 3×3 mm defect [11,21]. A 3-mm-diameter trephine bur with a depth of 3 mm was used to create circular defects surgically in the present study, considering the tibial thickness and fracture risks of the rats. The defects created in the control, PG/COL, and COL groups did not completely heal, whereas the defects treated by TQ almost fully healed after 28 days. Although the 3×3 mm defect model we used in our study seemed

inadequate for the groups using only a membrane, it appears to be a suitable model for the TQ groups. According to these findings, it would be more appropriate to use smaller-diameter defect models in GBR applications. However, this issue does not have an impact on the implications of these findings regarding the effect of TQ on bone healing. Analogously, Kütan et al. [11] obtained similar results, reporting new bone formation only in the GBR group.

GBR aims to exclude the proliferation of soft tissue cells to protect blood clots and cavities created using the barrier membrane [3]. Some authors have suggested that both membrane exposure and removal may cause incomplete bone healing; therefore, it has been suggested that the degradation period of membranes used in GBR should vary between 3 and 9 months. This is the period that is required to obtain bone formation [5]. However, despite the advantages of barrier membranes for bone healing, this technique also has disadvantages such as membrane instability in the application area if not fixed properly, and the possibility of wound edge opening. In such cases, the exposed membrane comes into contact with the oral flora, resulting in bacterial infection of the defect area and, therefore, impaired bone healing [5].

The colonization of bacteria may cause early degradation of COLs and adversely affect the procedure. *Fusobacterium nucleatum* and PG are 2 anaerobic Gram-negative bacteria that inhabit the oral cavity. These significant microorganisms play essential roles in the formation of mature multispecies biofilms on tooth surfaces [30,31]. Additionally, *F. nucleatum* and PG have virulence factors that trigger host inflammatory reactions, resulting in the destruction of periodontal tissues and the supporting bone [32]. In a study where Kütan and colleagues [11] imitated the exposure of the membrane by infecting the bone defects created in rat tibias; effective infection was achieved using a suspension of 10^9 CFU/mL PG. In the present study, infection of the defects was achieved using a similar method. The high tissue response and the observation of microbiological growth only in the PG group show that infection with PG may be achieved and that this experimental model may be suitable. Clinically, infection was observed only in the PG group. The reason why it was not observed in the other groups, especially in the PG/COL and PG/TQ/COL groups, can be explained by the fact that collagen creates a barrier and the membranes do not undergo lysis in the early period. Further studies using polymerase chain reaction for bacterial identification may contribute to differentiating the effects of TQ against infection from the effects of a single membrane.

In addition to the antioxidant, anti-inflammatory, antitumor, immunomodulatory, bronchodilatory, hypotensive, antidiabetic, hepatoprotective, gastroprotective, antihistaminic, and neuroprotective effects of NS, antimicrobial properties have also been described [15,16,33]. In a study that evaluated the effect of TQ on pathogenic bacteria, TQ exerted an anti-infection and resistance-changing effect. However, the antimicrobial properties of TQ are selective only for Gram-positive bacteria, whereas Gram-negative species are resistant [33]. Despite that, its efficiency was shown to be 4 times higher than that of tetracycline and benzalkonium chloride and therefore has been suggested for use in multi-drug resistant infections [33]. It has recently been shown that TQ exerts an antibacterial effect and has the potential to alleviate the virulence properties of *F. nucleatum* and PG. It has the ability to prevent biofilm formation and the hemolytic action of these bacteria. Thus, TQ is promising in terms of being developed and utilized for periodontal treatment, specifically for preventing periodontitis progression [34]. In this study, the bone-healing parameters in the TQ-containing groups were better than those in the other groups. Many reasons could be proposed for these findings, including the antimicrobial effect of TQ, which indirectly affects the inflammatory response. In addition, the microbiological examinations observed that the

membrane functioned as a barrier and could prevent bacterial colonization, regardless of its TQ content. However, for ethical reasons, this study included a small number of animals, and the sample may have been insufficient for microbiological examinations. These findings should therefore be interpreted as preliminary. Further studies are needed to investigate the antimicrobial effects of TQ when applied locally.

In experimental studies evaluating bone healing, a histological evaluation is required to understand the degree of healing at the cellular level. However, histomorphometric evaluations are accepted as the gold standard for direct and quantitative assessments of bone cells, activity, and bone matrix. In this study, newly formed bone and osteoblast counts in the bone defects in rat tibias were evaluated histomorphometrically, and the capillary density and tissue response were evaluated histologically. The findings of significantly higher newly formed bone amount, osteoblast number, and capillary density in TQ treated groups suggest that TQ had a positive effect on bone healing. Furthermore, the fact that these values were high in the PG/TQ/COL group suggests that the effect of TQ on bone healing is antimicrobial, even if the defect region is infected with *PG*. The results of this study are in line with the studies of Al-Hijazi and Mohammed, who reported that NS promoted bone healing by inducing the rapid generation of bone trabeculae and mature bone formation [35]. Al-Mutheffer [36] showed that epicutaneous administration of NS extract positively affected bone healing by increasing cell migration and differentiation processes, extracellular matrix formation, and extracellular matrix organization. An animal study performed on femoral bone fracture healing showed that continuous delivery of TQ may facilitate bone healing [37]. Another recent study showed that TQ expedited bone formation and reduced the retention period in rapid maxillary expansion [19].

TQ was also found to induce anabolic activity in osteoblastic cells [38]. Pratama et al. [39] examined socket healing in diabetic rats post-extraction and determined that TQ expedited the healing process by reducing blood glucose levels and inducing odontoblastic activity. Ezirganli et al. [40] investigated the effect of the systemic application of TQ on healing in CSDs by forming calvarial defects in osteoporotic rats. In the second- and fourth-week follow-ups, they observed acceleration of bone healing resulting from the systemic application of TQ. TQ has also been shown to induce bone healing by expediting osteoblast differentiation and BMP-2 activation in a study by Wirries et al. [38].

TQ has been shown to increase the amount of blood in the defect region and thereby increase angiogenesis, which plays a key role in bone healing by accelerating blood flow [41]. The study of Arslan et al. [21], which evaluated the effects of systemic TQ application on bone healing in rat tibia, reported that the capillary density in the newly formed bone was high, indicating that TQ increased vascularization and thus showed superior efficacy for accelerating bone defect healing. However, another possible explanation for the increase in capillary density may have reflected maintenance of the inflammatory phase at about a month after surgery.

New bone formation was found to be significantly higher in the TQ-treated groups in the present study. Therefore, it was concluded that TQ positively affected bone defect healing. The newly formed bone amount, osteoblast number, and capillary density in the TQ/COL group were higher than in the COL group, which suggests that TQ has positive effects in addition to the possible effects of the barrier membrane in bone healing. The absence of a significant difference in terms of new bone formation between the TQ groups supports the

view that even if the defect area is infected, the effectiveness of TQ on bone healing will not change. The extensive new bone formation and high number of osteoblasts in the TQ groups supports the view that TQ positively affects bone healing. Furthermore, capillary density in the TQ groups was high in the connective tissue surrounding the new bone trabeculae. It was suggested that TQ increased angiogenesis and thus has superior efficacy for accelerating bone defect healing.

Some limitations of the present study should be considered, such as the relatively low number of animals, which provided insufficient samples for microbiological evaluation, the use of CFUs instead of polymerase chain reaction, and the relatively small amount of quantitative analysis. Nevertheless, the study may serve as a supportive experiment to highlight the advantages of TQ on bone healing.

In light of the results obtained from this study, TQ-containing COLs appear to have positive effects on bone healing regardless of whether the bone defects are infected. In this study, the hypothesis that the TQ-containing COL may exert an antibacterial effect in the recovery of bone defects that are surgically infected with PG was demonstrated by microbiological examinations. Different studies utilizing more advanced study designs are warranted to evaluate the effects of TQ on bone healing in more detail. Furthermore, due to its antimicrobial effect and supportive effect on wound healing, adding TQ to a barrier membrane might be beneficial in the oral cavity, which contains microbial flora, when regenerative processes are planned.

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