ORIGINAL ARTICLE

Protective Effect of Quercetin Administration in Bacterial-induced Periodontitis on Rats

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ABSTRACT

Introduction: Quercetin is a flavonoid found in a variety of plants, including guava, apples, onions, and tea. It's been used as an anti-oxidant and anti-inflammatory substance for a long time. This study aims to investigate the effect of quercetin on periodontitis caused by Porphyromonas gingivalis-adhered ligatures. **Methods:** Eighteen male adult Sprague Dawley rats were divided into 3 groups, namely the control group (C, n=6) and the other two groups that received quercetin at 45mg/kg/day as a preventive (Qp, n=6) and a curative treatment (Qc, n=6), respectively. Under general anaesthesia, periodontitis was induced by placing a 3/0 non-resorbable sterile silk thread around the mandibular incisor teeth of eighteen male adult Sprague Dawley rats. The ligature placement caused severe irritation in the periodontal tissue. The animals were euthanized after 14 days of post-induction treatment, and samples of the mandibular portion were kept in formalin and prepared for histological processing to determine the grade of inflammation (GI). The periodontal pocket depth (PPD) was measured using the Michigan-O probe with Williams marks at the mesial and lingual sites of the rat's incisors tooth to determine the clinical parameter. **Results:** Qp showed the best improvement, in both parameters, clinically (PPD score, p=0,0018 at the lingual site, and p=0,0264 at the mesial site) and histologically (GI, p=0,0002). Significant differences were found in preventing clinical attachment-loss statistically (p<0,05) on Qp, better than the Qc at an equal dose (p<0,05). **Conclusion:** This finding suggests that quercetin administered as a preventive measure (Qp) may promote the healing process of gingiva in periodontitis conditions better than the control group and curative group (Qc).
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INTRODUCTION

As an infectious disease, periodontitis is associated with the presence of specific pathogenic bacteria that formed colonies in the subgingival area. *Porphyromonas gingivalis* and *Actinobacillus actinomycetemcomitans* are two microorganisms that are pathogenic to periodontal tissue. The occurrence of periodontal infection is influenced by several risk factors, both local and systemic. Local factors include the immune system, pre-existing disease and areas of plaque retention due to impaired restoration (1). Periodontitis is caused by bacterial infection and the immunological response of the host. Appropriate nutritional intake can enhance the immune response. Some beneficial phytochemicals are found in some plants, such as, tannins, alkaloids, essential oils, and flavonoids. They possess a variety of antimicrobial, anti-inflammatory, antibiotic, analgesic, and antioxidant properties (2). Quercetin is a bioactive molecule that is frequently employed in botanical medicine and traditional Chinese medicine due to its powerful antioxidant action (3). It is also commonly known as a polyphenolic flavonoid which can be found in several edible plant that are consumed on a daily diet. As an antioxidant, it can strengthen the membrane's resistance to oxidative damage caused by free radicals (4,5). Certain commercial products containing quercetin are used as dietary supplements to promote human health. Quercetin has been reported that it has antimicrobial activity against Gram-negative and Gram-positive bacteria (6-8). Previously published research established quercetin supplementation had a considerable influence on C-reactive protein, especially at levels larger than 500 mg/day (9).

A rapid release of reactive oxygen species (ROS) and an increase in polymorphonuclear (PMN) count characterize the pathophysiology of periodontal disease. This is evidenced by increased oxidative

stress in periodontal tissues. To prevent tissue damage induced by reactive oxygen species, periodontal tissues will require enough antioxidant levels under this state. As a result, antioxidants were necessary for the local or systemic therapy of periodontal disease (10). Xiong revealed that quercetin suppresses the production of IL-1, IL-6, IL-8, and TNF-α in Human Gingival Fibroblast (HGF) stimulated with P. gingivalis LPS via PPAR-γ activation (11). These findings are supported by the wei study, which showed that quercetin can enhance the antioxidant capacity of periodontal ligament cells and protect them from oxidative stress damage by activating the NRF2 signaling pathway, consequently preventing alveolar bone loss in periodontitis (12). According to several research, periodontal disease has been linked to a range of systemic disorders, including rheumatoid arthritis, cardiovascular disease, and diabetes (13). In periodontitis states, the tooth's transgingival position can impede wound healing, even under ideal conditions. The formation of a long junctional epithelium, the maturation of gingival connective tissue, and the limited regeneration of alveolar bone and cementum were indicating to wound healing. Inflammation, granulation tissue creation, and matrix formation and remodeling are the three phases of healing processes that are overlapping. By following periodontal regenerative procedures, we can assess clinically or histologically to identify those stages and thus determine the outcome of the healing process (14).

Despite the fact that quercetin has been established in studies, its information on periodontitis is still limited. This study aimed to determine the efficacy of quercetin to accelerate the gingival wound healing by reducing the inflammation caused by periodontitis, which can be observed through clinical and histological evaluation. We hypothesized that the quercetin's antimicrobial and antioxidant properties would aid in host immunity and tissue regeneration at a wound formed during periodontitis development.

MATERIALS AND METHODS

The experimental design and protocols were reviewed and approved by the Bandung Institute of Technology's ethics committee (no.02/KEPHP-ITB/12-2017) in Bandung, West Java-Indonesia.

Animal and drugs treatment

Animal Laboratory and Research Unit at Bogor Agricultural University, Indonesia, provided healthy male Sprague Dawley rats (170–180 g). Eighteen rats were housed and adapted two weeks prior to the experimental period in an animal laboratory room with air conditioning (22°C-23°C) with a 12-hour light/dark cycle. The animals were given a diet of commercial standard rodent pellets and ad libitum water. All animals were treated humanely according to The National Academy of Science's Guide for the Care and Use of Laboratory Animals, 8th Edition 2011. All of the experiments were carried out in the Experimental Animal Laboratory of School of Pharmacy at Bandung Institute of Technology. Trial's Flow diagram are shown in Figure 1. The animals were randomly separated into three groups: one received vehicle (C) as a control group (n=6), and the others were treated with quercetin suspension at 45 mg/kg/day in CMC-Na 0,5% (equal to a dosage of 500 mg in humans). The suspension was given in two treatment types: preventative (Qp, n=6) and curative (Qc, n=6). The Quercetin (Sigma, St. Louis, M.O., USA) material is obtained from an authorized supplier of Sigma Industries in Indonesia. Animals with abnormal habits (twirling and limping) were excluded. Quercetin was administered orally, one week before the induction began for the preventive group. In comparison, the curative group (Qc) started their treatment one week after the induction. Quercetin administration for all groups was continued until 2 weeks post-induction.

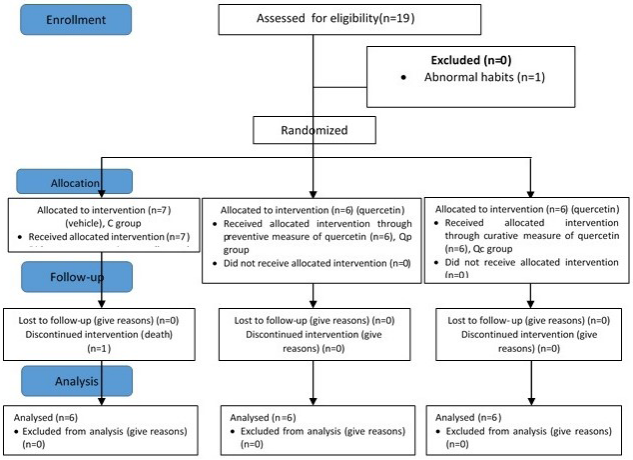

Figure 1: Trial's Flow Diagram

Wound models

The wounding was performed by separated the gingival tissue from the tooth surface then enabling to insert the ligature into the incisive mandibular sulcus of rats under general anaesthesia. Before application, the ligature was made by immersing it for three seconds into P. gingivalis (ATCC 33277) suspension. The bacterial suspension was prepared by culturing P. gingivalis in BHI media, which was previously incubated for three days at 37 C. The absorbance of the microbial suspension was measured using a UV-Vis spectrophotometer at a wavelength of 625 nm. Then the bacteria were diluted to obtain an absorbance between 0.08-0.13 or equivalent to 0.5 McFarland. The gingival tissue was separated from the tooth surface by a surgical blade no.15 to enable inserting ligature into the incisive mandibular sulcus at the mesial site of the target teeth. The wound is made in the form of a perpendicular incision made by the same person. Before treatment, all rats were confirmed to have no periodontal pockets yet to maintain sample homogeneity. The procedure was carried out under general anaesthesia with a (1:1) mix solution of ketamine

10% and xylazine 2% (0.1 ml/100 g, i.p). The bacterial-adhered ligatures were used to accelerate periodontitis state, which leads to forming a pocket between teeth and gingiva margin of the rats. On the 14th-day post-induction, the animal has euthanized. The PPD was assessed, and the mandibula part was taken and stored in formalin for histology analysis. The clinical parameter was evaluated by measuring the periodontal pocket depth (PPD) using the Michigan O probe with Williams markings at mesial, distal, and lingual sites of rats' incisive mandibular teeth from the gingiva's edge to the bottom of the pocket. However, the distal site did not show any pocket changes, so that the only depth measured in the mesial and lingual areas was continued in the analysis (Figure 2).

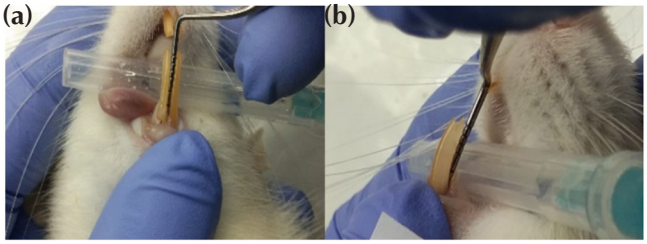

Figure 2: Probing technique in PPD assessment at the mesial site (a) and lingual site (b).

Histological evaluation

Gingival tissues were extracted from the mandibula after it had been cleansed from muscle and connective tissue, then fixed in 10% neutral buffered formalin for 72 hours and followed by decalcified for 40 days in a 10% EDTA solution. The gingival tissue was cut longitudinally and dehydrated in successive baths of Isopropyl alcohol (70%, 90%, 95%, and 100%), clarified in xylene, and embedded in paraffin wax after decalcification was accomplished. A rotary microtome was used to cut several tissue sections at 4 µm thickness from each paraffin block. Afterwards, the tissue sections were stained with Masson-trichome for fiber collagen examination under an Olympus BX41 microscope with 400x magnification for assessing the Grade of inflammation histologically. This procedure done by an anatomical pathologist to ensure consistency of assessment. The inflammation

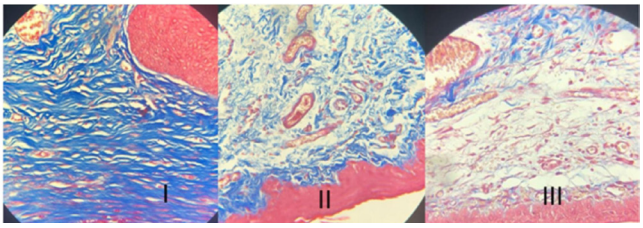

Figure 3: Scale of inflammation I-III on the rat's periodontal tissue through histological observation using Masson's trichrome staining with a magnification of 400x

grades were categorized based on the fibro-collagen density indicated by the colour blue of Masson's trichome staining as seen as Figure 3. This staining is used to see the density of fibro-collagen that is formed in the tissue during the healing process. It can be seen from the visible intensity of the blue colour. The healing process is characterized by thick fibro-collagen with high blue intensity, we give a score of 1, while the light blue represents severe inflammation, we give a score of 3 on a scale of 3.

Statistical analysis

The data were expressed as mean ± SD which analyzed using repeated measures of variances using GraphPad Prism 8.0.2 software. When Kruskal-Wallis indicated a significant ($p<0.05$) at a 95% confidence level, Dunn's multiple comparisons test was employed to test for differences between means.

RESULTS

As illustrated in Figure 4, periodontal pocket depths (PPD) and inflammation grade scores were assessed and recorded. Figure 4 shows that on the mesial side, the greatest improvement was shown by the Qp group. This indicates the protective effect of quercetin, which can increase tissue repair in wounds formed in the mesial portion of the tooth. For statistical analysis, Kruskal-Wallis was used. For each group, values are expressed as mean ± standard deviation with n=6 animals. Kruskal-Wallis analysis was used to determine the p-values for mesial PPD, lingual PPD, and inflammation grade score, respectively, which were 0.0264; 0.0018; and 0.0002.

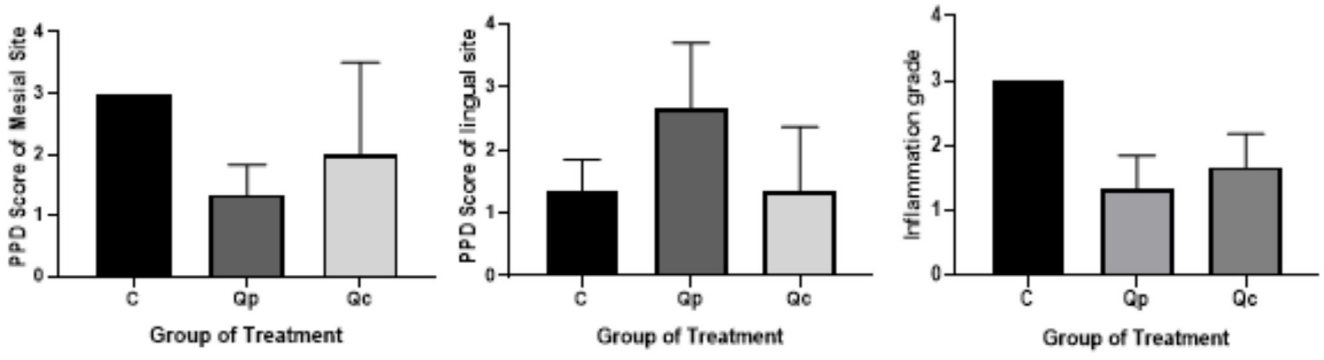

Figure 4: Profile of PPD mesial (a), PPD lingual (b), Inflammation grade (c). C: Control group, Qp: Preventive group, Qc: Curative group

The Qp and control groups had a significant difference (adjusted P-value= 0.0123), according to Dunn's multiple comparisons test, indicating a significant increase in wound-healing activity. The results indicated that there are significant differences in inflammation grades between groups as a result of treatment. Additionally, the PPD score reflects a significant effect of quercetin during treatment. The distribution of inflammation scores based on the histological score in Figure 3 is shown in Figure 4c. The Qp group was found to have the lowest PPD score following treatment.

The result indicates that quercetin in their therapeutic equivalent doses had promoted the wound-healing in Qp group when given as pre-treatment. It can be seen from PPD reduction of the rat's mandibular central incisor teeth in the Qp group compared with the control group.

DISCUSSION

The approach adopted in this research is a modification of an established induction technique. Previously published research used lipopolysaccharide (LPS), a component of P. gingivalis, as the inducer (11). However, in our investigation, we used P. gingivalis bacteria embedded in ligature as the inducer. Additionally, we did not conduct cell cytotoxicity assays since we converted the quercetin dosages found in available commercially 500mg quercetin supplements, so it was assumed that the quercetin doses were within a safe dosage range. The differences may occur as a result of the lingual location's anatomical structure. This condition facilitated plaque accumulation on the incisors, creating an ideal environment for pathogen bacteria to thrive. Xiong demonstrated that dental plaque and the host immune response both contribute to periodontitis pathogenesis. Periodontal tissue damage occurs primarily as a result of inappropriate host's response to dental plaque (15). As a result, a chronic inflammatory state persists, with inflammatory cells, primarily polymorphonuclear lymphocytes, releasing free oxygen radicals. Dental plaque accumulation can exacerbate gingival inflammation and result in gingival recession/decrease and deepening of the gingival groove, resulting in the formation of periodontal pockets. This process is accompanied by damage to the alveolar bone that supports the teeth, which can result in tooth instability. This destructive process is aided further by periodontal pathogenic bacteria' enzymes and by-products, which can degrade the host cell membrane and extracellular matrix (16,17). Without intervention, this condition will result in a slower healing process. The addition of quercetin enhanced its ability to regenerate cells. Previous research has established that quercetin's antioxidant activity is critical for wound healing because it prevents oxidative damage and promotes tissue repair (2). For improvements in lingual PPD, statistically significant differences in outcomes were established. Quercetin acts as an antioxidant by chelating metal ions that can form oxygen free radicals. Quercetin also exerts an effect on enzymes that regulate a variety of cellular functions, including mast cell histamine secretion. The release of arachidonic acid from phospholipids in the cell membrane is then catalyzed by phospholipase. Thromboxane, inflammatory prostaglandins, and leukotrienes all require arachidonic acid as a substrate. It also blocks the enzymes cyclooxygenase and 5-lipooksigenase, which catalyze the conversion of arachidonic acid to its metabolites (18). According to Xiong, quercetin acts as an anti-inflammatory by inhibiting the production of IL-1β, IL-6, IL-8, and TNF-α by activating PPAR-γ which then suppresses the activation of NF-κB (13).

Another element that also plays an important role, called fibroblasts. In Figure 2, fibroblasts are shown in blue colour. Taskan revealed that there was an improvement in wound healing after quercetin administration in preventing inflammation. Its recovery indicated by decreased inflammatory cells and increased fibroblast cells in his study (19). Fibroblasts play a role in wound healing by breaking down fibrin clots, forming new extracellular matrix (ECM), and forming collagen structures to support other cells. The ECM composition has an effect on the fibroblast migration activity at the wound site. At the wound site, the fibroblast migration activity is influenced by the ECM composition. When there is damage to the ECM, fibroblasts will proliferate and then adhere to the fibrin clot and wound bed via several integrins. They then proliferate to produce matrix metalloproteinases (MMPs) and other proteinases, such as separinase, to remove denatured protein residue and matrix material that is no longer needed after the wound has healed. Fibroblasts also produce proteinase, a tissue metalloproteinase inhibitor (TIMPS), which strictly controls this process. At the same time, a new ECM was generated concomitantly with fibronectin and hyaluronic acid. Collagen III is rapidly produced, with an initial matrix that acts to inhibit pathogens and prevent serum and fluid loss. Furthermore, collagen III will be degraded by proteases and remodeling by fibroblasts into collagen type I, which has a much higher attractive strength but takes longer to settle (20).

In an experimental periodontitis model, quercetin treatment can improve osteoblast activity while decreasing osteoclast activity, apoptosis, and inflammation, lowering the risk of alveolar bone loss (19,21). As reported before, antioxidant properties of quercetin may be contributed in reducing lipid peroxidation, preventing the tissue damage and promoting vascular improvement, concomitantly. Preventive administration of quercetin has been shown to accelerate the healing process. In this case, quercetin can enhance the work of antioxidant enzymes and downregulates the NF-κB, which leads to inhibit the expression of MMPs and TSLP secretion (22). MMP-2

and MMP-9 are responsible for the degradation of gelatin in the extracellular matrix and type IV collagen in the basement membrane, according to Chan's research. As a result, invasion of bone will be more straightforward. In his study, giving Quercetin (10 μM) was shown to suppress MMP-2 and MMP-9 expression and proteolytic activity (23). The limitation of this study is the quercetin preparation is still used in suspension form with 0.5% NaCMC solution as a vehicle. However, the stability of quercetin in the preparation is low. It is necessary to have its bioavailability in the blood after administration to determine the blood plasma levels of quercetin. Hopefully, through accurate pharmacokinetic data, the effect of quercetin can be predicted and optimized to use in periodontitis therapy.

CONCLUSION

This study shows the presence and activities of quercetin in promoting wound healing is well established. Quercetin administration may have a protective role during periodontitis conditions. It is suggested that quercetin was able to reduce inflammation through antioxidant and anti-inflammatory activity. Supplementation of quercetin can act as a prophylaxis against free radical damage and products produced by pathogenic microbes. The impact of this study can be used as a reference for natural ingredients containing quercetin related to antioxidant activity with relevant mechanisms.

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