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Effectiveness of oral irrigation with an extract of green microalga *Nannochloropsis oculata* as an anti-inflammatory in rats infected with *Aggregatibacter actinomycetemcomitans*

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Abstract. *Revianti S, Andriani D, Parisihni K, Wahjuningsih E, Widyastuti. 2020. Effectiveness of oral irrigation with an extract of green microalga* Nannochloropsis *oculata as an anti-inflammatory in rats infected with* Aggregatibacter actinomycetemcomitans. *Biodiversitas 21: 2977-2981.* Aggressive periodontitis is strongly correlated with *Aggregatibacter actinomycetemcomitans*, a periodontopathic bacterium, which releases endotoxins and lipopolysaccharides (LPS). LPS acts as a stimulus to a variety of host cells that can promote the expression of pro-inflammatory cytokines, such as the tumor necrosis factor-α (TNF-α), in periodontal disease. The marine green microalga *Nannochloropsis oculata* has anti-inflammatory properties. This study aimed to examine the anti-inflammatory efficacy of oral irrigation with *N. oculata* extract against periodontitis induced by *A. actinomycetemcomitans* in a rat model. Twenty-four male Wistar rats were divided randomly into four groups (n = 6). First control group was without any treatment, the second to fourth groups were infected with *A. actinomycetemcomitans* and then orally irrigated with *N. oculata* extract at concentrations of 2.375%, 2.5%, and 2.625%. The mandibles of the euthanized rats were hemisected to measure the expression of TNF-α, interleukin-10 (IL-10, an anti-inflammatory cytokine), and osteoprotegerin (OPG) by immunohistochemistry. The first group (control) showed significantly higher expression of TNF-α and significantly lower expression of IL-10 and OPG compared to the other groups. Oral irrigation with *N. oculata* extracts reduced the expression of TNF-α and enhanced the expression of IL-10 and OPG in rats infected with *A. actinomycetemcomitans*. The effective concentration of *N. oculata* extract as an anti-inflammatory for oral irrigation was 2.375%.

Keywords: *Aggregatibacter actinomycetemcomitans*, anti-inflammatory, green microalgae, oral irrigation, *Nannochloropsis oculata*

INTRODUCTION

Periodontitis is an oral disease widespread among people from developing countries. Epidemiological studies show that poor oral hygiene is associated with high prevalence and severity of periodontal disease (Lertpimonchai et al. 2017). The prevalence of periodontal disease increases from the age of 40 (Wu et al. 2016; Nazir 2017). Periodontitis is inflammation of periodontal tissue initiated by oral microbial biofilms leading to the destruction of the connective tissue attachment. It is the most common disease of periodontal tissue. Untreated periodontal disease could develop into periodontitis and cause damage to the periodontal support tissue, including connective tissue, periodontal ligament, and alveolar bone (Wu et al. 2016; Van Dyke 2008). Plaque bacteria on the surface of the teeth are the major cause of periodontitis. *Aggregatibacter actinomycetemcomitans* is the bacterial cause of aggressive periodontitis characterized by progressive periodontal tissue damage (Newman et al. 2011).

The bacterial plaque components, lipopolysaccharides (LPS) and lipoteichoic acid, interact with toll-like receptors on epithelial cells, leukocytes, and fibroblasts, and stimulate cytokine production. Cytokines play a crucial role in the maintenance of healthy periodontal tissue (Newman et al. 2011; Murrat and Wilton, 2003). Tumour necrosis factor-α (TNF-α), a pro-inflammatory cytokine with an important role in the immune system, induces bone resorption (Singh et al. 2014). Inflammation of the periodontium leads to the destruction of ligament and alveolar bone via osteoclasts which are major bone resorption cells that differentiate from monocytes or macrophage precursors under the regulation of the critical cytokine macrophage colony-stimulating factor and RANK ligand (RANKL). Osteoprotegerin (OPG) is a decoy receptor for RANKL and reduces osteoclastogenesis and bone resorption. TNF-α, interleukin-1 (IL-1,) and PGE2 also promote osteoclast activity, particularly in the inflammatory osteolysis state in the pathogenesis of periodontitis (Hienz et al. 2014). Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine, which contributes to the maintenance of bone mass through inhibition of osteoclastic bone resorption and regulation of osteoblastic bone formation (Zhang et al. 2019).

Periodontal disease is caused by bacterial infection; therefore, antibiotic treatment administered systemically or locally may be appropriate (Newman et al. 2011). The commonly used antibiotics are tetracycline, metronidazole, amoxicillin, clindamycin, and ciprofloxacin (Prakasam et

al. 2012). Minocycline, in the form of mouthwash or gel, has been shown to reduce periodontal pocket depth (Augustina 2010). However, antibiotics can cause various side effects, such as bacterial resistance, allergic reactions, toxic reactions, and tooth discoloration (Heta et al. 2018). The goal of periodontal disease therapy is to eliminate gingival inflammation, reduce pocket depth, and increase attachment (Newman et al. 2011). Recently, the concept of therapy has begun to change, as demonstrated by research on host responses to bacteria that make a major contribution to the pathogenesis of periodontal disease (Newman et al. 2011; Ebersole et al. 2013). The role of the host response in the inflammatory process and the development of tissue damage in periodontal disease is the basis for a therapeutic approach that inhibits pro-inflammatory mediators involved in the response of damaged tissue (Ebersole et al. 2013). Non-steroidal anti-inflammatory drugs (NSAIDs), such as indomethacin, flurbiprofen, and naproxen, administered daily for three years in periodontal therapy can significantly slow the rate of alveolar bone loss compared to placebo. NSAIDs are widely used for various diseases in adults. Side effects can range from mild to severe, including erosion, ulceration, haematemesis, melaena, or perforation (Wongrakpanich et al. 2018). Therefore, it is necessary to develop a natural anti-inflammatory drug that is expected to minimize side effects.

Marine green microalgae produce secondary metabolites such as alkaloids, flavonoids, glycosides, terpenoids, and phenazines. In addition to secondary metabolites, algae contain proteins, carbohydrates, lipids, polysaccharides, polyols, and phycobiliproteins. Many of these secondary metabolites are used in various health food sectors. A study of the anti-neuroinflammatory capacity of green seaweed extracts from Malaysia showed a reduction in inflammatory mediators like NO, TNF-α, IL-6, and IL-1β (Barbalace et al. 2019). Microalgal extracts exert an anti-proliferative effect and increase IL-10 in sheep peripheral blood mononuclear cells (Ciliberti et al. 2019). These marine green algal extracts also prevented osteoporosis via both suppression of osteoclast differentiation and accelerated osteoblast formation in separate in vitro experiments (Venkatesan and Kim 2011).

Nannochloropsis oculata is a green microalga that is nonmotile, non-flagellated, and round in shape, with a diameter of 2–4 μm (Kagan et al. 2015). It has potential because of its high growth rates and ease of cultivation even under unfavorable environmental conditions. It is also a novel source of important bioactive compounds such as antioxidants, proteins, vitamins, minerals, soluble dietary fiber, polyunsaturated fatty acids, polysaccharides, sterols, carotenoids, tocopherols, terpenes, phycobilins, hydrocolloids, and phycocyanins (Sathasivam 2019). It has bioactive compounds able to minimize the production of free radicals and enhance antioxidant strength (Borges et al. 2011; Yanuhar et al. 2011), and is reported to have high levels of proteins, as well as flavonoids, tannins, glycosides, alkaloids, and saponins, which are beneficial for lowering cholesterol, and can be developed into functional food ingredients (Fithriani and Ambarwaty 2020)

An in vitro study by Revianti and Kristanti (2013) showed that *N. oculata* extract is non-toxic to fibroblast stem cells up to a threshold concentration of 2.5%, while above this concentration it was toxic. Kafaie et al. (2012) reported non-toxic effects of *N. oculata* at 12 g/kg of body weight (bw) per day in an acute toxicity study and 6 g/kg of bw in a sub-chronic toxicity study of rats. Therefore, our study aimed to determine the efficacy of *N. oculata* extract as a natural anti-inflammatory drug in rats infected with *A. actinomycetemcomitans* by measuring the expression of TNF-α, OPG, and IL-10 in periodontal ligaments. We hypothesized that treatment with *N. oculata* extract would reduce TNF-α expression and increase OPG and IL-10 expression in periodontal ligament tissue.

MATERIALS AND METHODS

Nannochloropsis oculata

N. oculata was obtained in the form of dry powder from Balai Perikanan Budidaya Air Payau, Situbondo, Jawa Timur, Indonesia, and stored at −20°C until use (Nuño 2013).

Experimental animals

Experiments were performed using 3-month-old adult male Wistar rats (180–200 g) obtained from the Biochemistry Laboratory, Faculty of Medicine, Airlangga University, Indonesia. The animals were acclimatized to laboratory conditions at room temperature before experimentation. They were housed in 40 cm × 30 cm × 14 cm plastic cages with soft bedding, with six animals per cage, under standard conditions (12 h light/dark cycle at 25 ± 2 °C, with enough air). They were fed a standard diet and provided water ad libitum. All the experiments were carried out between 07:00 and 15:00 (Bryda 2013). The experimental protocol was approved by the Animal Ethics Committee of the Faculty of Dentistry, Universitas Hang Tuah, Surabaya, Indonesia.

Figure 1. Green microalga *Nannochloropsis oculata* dry powder and solution

Experimental design

We used a post-test-only control group design, a type of true experimental design, in this study. The sample size was 24 rats which were divided into four groups. The sampling technique was simple random sampling. After preparing the rats according to the sampling criteria on the 1st day, the 24 rats were acclimatized for 7 days in cages. Then on the 7th day, the rats were divided into four groups, namely 1st, 2nd, 3rd, and 4th groups, and marked. Each group consisted of six rats placed in one cage.

***Aggregatibacter actinomycetemcomitans* infection**

The rats were housed in pairs under specific pathogen-free conditions. They were weighed once a week to ensure proper growth and nutrition. Injections were given three times each week for 8 weeks. First, anesthesia was induced with 4–5% isoflurane and maintained with 1–2% isoflurane. All rats received a total volume of 2 µl of the solution via a 33-gauge syringe to the lingual interproximal gingiva between the first, second, and third mandibular molars (Dunmyer et al. 2012). The 1st group (control) received neutral 1× phosphate-buffered saline (PBS), whereas the 2nd, 3rd, and 4th groups received *A. actinomycetemcomitans* LPS 1×10^9 CFU which was diluted in 10 µg/µl of PBS with a micropipette.

Preparation of irrigation material from *Nannochloropsis oculata* and method of oral irrigation

N. oculata biomass was crushed into a powder in an electric blender (Figure 1). The irrigation solution was prepared by dissolving *N. oculata* powder in 0.2% sodium carboxymethylcellulose (Na-CMC) and made up to concentrations of 2.375%, 2.5%, and 2.625%. On the 12th day, the buccal and lingual parts of the first, second and third molars of the lower jaw were irrigated in rats in the 2nd, 3rd, and 4th groups with 0.14 ml of the 2.375%, 2.5%, and 2.625% concentrations of the *N. oculata* extract, respectively. Oral irrigation continued once daily for 25 days.

Collection of samples

At the end of the experiment, the rats were withheld food overnight, subjected to anesthesia using thiopental (Thiopentax 0.5 g, 20 mg/kg) and sacrificed. The mandibles were hemisected, and posterior block sections were immersed directly in a 10% neutral buffered formalin fixative solution for 72 hours (de Araujo Junior et al. 2013) to measure the expression of TNF-α, IL-10, and OPG by immunohistochemistry.

Immunohistochemical analysis

The immunohistochemical analysis and the histological scoring of the periodontal ligament tissues were conducted by two oral pathologists. The sectioning was performed in the laboratory of Pathology Anatomy and subsequently analyzed by light microscopy in the Department of Pathology Anatomy, Faculty of Dentistry, Universitas Hang Tuah, Indonesia. Specimens were fixed in 10% neutral buffered formalin and demineralized in 5% nitric acid. Specimens (4 µm) were transferred to gelatin-coated slides, deparaffinized, and then rehydrated. Periodontal ligament tissue slices were then washed with 0.3% Triton X-100 in PBS, and endogenous peroxidase was quenched following incubation with 3% hydrogen peroxide. Sections were then incubated with primary antibodies, specific to TNF-α, OPG, and IL-10, at a 1:400 dilution overnight at 4 °C. After washing with PBS, slices were then incubated with the secondary antibody for 30 min, and immunoreactivity to TNF-α, OPG, and IL-10 was visualized using a colorimetric-based detection kit following the manufacturer's protocol (TreekAvidin-HRP Label+Biocare Medical Kit, Dako, USA). Sections corresponding to the area between the first, second and third mandibular molars were evaluated by light microscopy (400× magnification). TNF-α expression was quantified in macrophage cells, IL-10 expression in lymphocyte cells, and OPG expression in osteoblast cells found in periodontal ligaments. The expression in these cells was identified based on the brownish discoloration of the cytoplasm as a positive reaction to the monoclonal antibodies, namely anti-TNF-α, anti-IL-10 and anti-OPG, observed through light microscopy by two observers (de Araujo Junior et al. 2013).

Statistical analysis

The data are presented as mean ± standard error (SE) in the table and figure. All data were analyzed using one-way analysis of variance (one-way ANOVA), followed by the least significant difference (LSD) test, with SPSS version 17; $p < 0.05$ indicated a statistically significant difference.

RESULTS AND DISCUSSION

None of the animals died as a result of experimental treatment up to the last day of the study. Expression of TNF-α, OPG, and IL-10 in the periodontal ligament is given for all groups in Table 1 and Figure 2.

The Shapiro–Wilk test of normality was performed for each group because the number of samples was less than 50; test results indicated that the distribution of the data was normal. The results for Levene's test results indicated that the data had homogeneous variance ($F = 0.861$, $p > 0.05$). Therefore, we proceeded with ANOVA. The results of ANOVA and the LSD test were significant ($p < 0.05$), meaning that there were significant differences between groups in TNF-α, OPG, and IL-10 expression.

The expression of TNF-α in periodontal ligament tissue in the 1st group was significantly higher than that in the 2nd, 3rd, and 4th groups ($p < 0.05$). TNF-α expression in the 2nd group was significantly lower than that in the 3rd and 4th groups ($p < 0.05$), and expression in the 3rd group was significantly lower than that in the 4th group ($p < 0.05$). The expression of OPG and IL-10 in the 1st group was significantly lower than that in the 2nd, 3rd, and 4th groups ($p < 0.05$). OPG and IL-10 expression in the 2nd group was significantly higher than in the 3rd and 4th groups ($p < 0.05$), and expression in the 3rd group was significantly higher compared to the 4th group ($p < 0.05$).

Table 1. Expression (mean ± SD) of TNF-α, OPG, and IL-10 in periodontal ligament tissue of rats treated with *Nannochloropsis oculata* extract.

Group	TNF-α			OPG			IL-10		
1st group (control group treated with placebo)	13.33	±	1.86	6.83	±	1.72	5.33	±	1.03
2nd group (irrigation with 2.375% *N. oculata* extract)	4.50	±	1.64	16.00	±	2.19	14.50	±	2.43
3rd group (irrigation with 2.5% *N. oculata* extract)	6.17	±	1.47	12.67	±	2.80	12.00	±	4.29
4th group (irrigation with 2.625% *N. oculata* extract)	8.33	±	1.97	7.67	±	1.21	7.50	±	1.87

Periodontitis is a disease of the oral cavity which is ranked second among the major health problems in Indonesian society (Wu et al. 2016). Periodontitis causes progressive periodontal tissue damage. If not properly treated, it can progress to the bone destruction stage, causing tooth loss. The cause of periodontitis is anaerobic bacteria, including *A. actinomycetemcomitans*, which is now known as the main pathogen in aggressive periodontitis. The treatment for periodontitis can be nonsurgical, surgical therapy or a combination of both, accompanied by antimicrobial treatment (Newman et al. 2011). The increasing antibiotic resistance in bacteria has encouraged researchers to find new antibacterial compounds in the highly diverse Indonesian marine biota; one such species is the green microalga *N. oculata*. We determined the effectiveness of different concentrations of *N. oculata* extract to reduce TNF-α expression and to increase OPG and IL-10 expression in periodontal ligaments of rats infected with *A. actinomycetemcomitans*.

The results obtained for periodontal ligament tissue showed that the expression of TNF-α in the group infected with *A. actinomycetemcomitans* was significantly higher than in the treatment groups ($p < 0.05$). The expression of OPG and IL-10 in the group infected with *A. actinomycetemcomitans* was significantly lower than in the treatment groups ($p < 0.05$). This indicates that *A. actinomycetemcomitans* causes aggressive periodontitis, which can lead to progressive damage. *A. actinomycetemcomitans* release LPS and stimulates inflammatory cells in periodontal tissue to produce cytokines, such as IL-1, IL-6, IL-8, TNF-α, and prostaglandin E2 (PGE2). These cytokines cause the production of matrix metalloproteinases (MMPs) to increase, which can lead to extracellular matrix damage in periodontal tissue (Herbert et al. 2016), thereby causing osteoclasts to be activated. Increased osteoclast activity accompanied by continuous extracellular matrix damage can trigger periodontal tissue damage which causes bone resorption (Maduratna and Setiawatie 2014). Pro-inflammatory cytokines, including TNF-α, IL-1, and IL-6, that trigger pro-resorptive actions are highly upregulated by *A. actinomycetemcomitans*, and thus promote osteoclast formation and bone resorption (Swastini et al. 2019). *A. actinomycetemcomitans* infection leads to increases in the pro-inflammatory cytokines TNF-α, IL-17, and RANKL, and decreases in the anti-inflammatory cytokines IL-10, TGF-β, and OPG (Izawa et al. 2014; Araujo-Pires et al. 2015; Levy-Ontman et al. 2017).

In some cases, antibiotic therapy is given to overcome factors that are not addressed by mechanical therapy. Antibiotics that cannot be regulated can cause residue to build up in tissue leading to resistance and possible poisoning; therefore, they can be harmful to humans (Heta et al. 2018; Augustina 2010). Consequently, to avoid these side effects, therapies need to be developed from natural ingredients that have good antibacterial power, one of which is the green microalga *N. oculata*.

Our results showed that rats infected with *A. actinomycetemcomitans* in the 2nd, 3rd and 4th groups treated topically with *N. oculata* extract at concentrations of 2.375%, 2.5%, and 2.625%, respectively, for 25 days showed a significant reduction in TNF-α expression and a significant increase in OPG and IL-10 expression compared to the 1st group. This demonstrates that *N. oculata* contains active compounds with anti-inflammatory properties.

N. oculata contains alkaloids, flavonoids, glycosides, terpenoids, and phenazines. It also contains proteins, carbohydrates, lipids, polysaccharides, polyols, and phycobiliproteins, etc. *N. oculata* is a known source of important bioactive compounds such as antioxidants, proteins, vitamins, minerals, soluble dietary fiber, polyunsaturated fatty acids, polysaccharides, sterols, carotenoids, tocopherols, terpenes, phycobilins, hydrocolloids, and phycocyanins (Sathasivam et al. 2019). A study assessed the anti-neuroinflammatory capacity of green seaweed extracts from Malaysia that reduced the elevation of inflammatory mediators like NO, TNF-α, IL-6, and IL-1β (Barbalace et al. 2019). Polysaccharides (PSs) produced by microalgae have been reported to exhibit anti-inflammatory bioactivity by interfering with TNF-α-induced inflammation in human coronary artery endothelial cells (Sayuti 2015). Microalgal extracts exert an anti-proliferative effect and increase IL-10 in sheep peripheral blood mononuclear cells (Ciliberti et al. 2019). These marine green algal extracts also prevented osteoporosis via both suppression of osteoclast differentiation and accelerated osteoblast formation in separate in vitro experiments (Venkatesan and Kim 2011).

In this study, we showed that oral irrigation with *N. oculata* extract at concentrations of 2.375%, 2.5%, and 2,625% for 25 days caused a significant reduction in TNF-α expression and a significant increase in OPG and IL-10 expression. The lowest TNF-α expression and the highest OPG and IL-10 expression were found in the 2nd group treated with the 2.375% concentration. This shows that the lowest concentration is the most effective in anti-inflammatory therapy.

This study shows that the higher the concentration of the treatment, the lower its effectiveness. At higher

concentrations, viscosity increases. The viscosity of a solution is inversely proportional to its fluidity; therefore, the lower the fluidity, the lower the ability of the active substance to spread and come into contact with the skin. If topical medication is easily spread on the surface of the skin, then the absorption of active ingredients will increase. The absorption of topical drugs has an important role in determining its effectiveness (Sayuti, 2015; Yanhendri, 2012).

In conclusion, oral irrigation with extracts of the green microalga *N. oculata* reduces TNF-α expression and increases IL-10 and OPG expression in rats that are infected with the *A. actinomycetemcomitans* bacterium. The effective concentration of the *N. oculata* extract as an anti-inflammatory for oral irrigation was 2.375%.

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