

Lemuru fish oil gel as host modulation therapy in periodontal ligaments induced with *Porphyromonas gingivalis*

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ABSTRACT

Background: Periodontitis affects approximately 20%–50% of the global population and is caused by gram-negative bacteria, such as *Porphyromonas gingivalis* (*P. gingivalis*). Host modulation therapy (HMT) is part of a periodontal therapy that is used as an adjunct to conventional periodontal treatment to reduce tissue damage. Lemuru fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can reduce the formation of matrix metalloproteinase species (MMPs) and will further increase the number of fibroblasts thereby stimulating collagen formation. **Purpose:** To determine the effect of lemuru fish oil gel on the collagen density and width of the periodontal tissue induced by *P. gingivalis* and the correlation between these parameters. **Methods:** Thirty male Wistar rats were divided into five groups. Induction of *P. gingivalis* was carried out first, then lemuru fish oil gel was applied to the gingival sulcus for 14 days, according to collagen scores in histological preparations using Masson's trichrome (MT). The width of the periodontal ligament was measured with an image raster program in μm . The data were analysed using statistics to test hypotheses using statistical product and service solutions (SPSS) version 24. **Results:** Significant differences in the results of the collagen density were observed between groups K- and K+ and groups K+ and P2. Meanwhile, no significant difference was observed between groups K- and P2, P3, P2 and P3 and K+ and P1. The mean values of the periodontal ligament widths were $299.61 \pm 51.82\mu\text{m}$ (K-), $425.85 \pm 61.54\mu\text{m}$ (K+), $346.93 \pm 33.53\mu\text{m}$ (P1), $370.15 \pm 49.42\mu\text{m}$ (P2) and $379.6 \pm 49.26\mu\text{m}$ (P3). **Conclusion:** Lemuru fish oil can affect the width of the ligament and the collagen density with an optimal concentration of 20%. The correlation between the collagen density and the periodontal ligament width was negative and not significant.

Keywords: Collagen density; lemuru fish oil; periodontitis-width of the periodontal ligament

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INTRODUCTION

Periodontal disease is a common issue, as seen in the Basic Health Research data published in 2018, which recorded the prevalence of periodontal disease in Indonesia reaching 73.1%–75%. Chronic periodontitis has been found to be one of the most widespread diseases among Indonesians.¹ The main factor causing periodontitis is anaerobic negative plaque bacteria. One of the most dominant bacteria found in chronic periodontitis is *Porphyromonas gingivalis* (*P. gingivalis*).²

P. gingivalis bacteria secrete endotoxin lipopolysaccharides (LPS), which can stimulate the activation of lymphocyte B cells to produce antibodies and stimulate the excretion of mediators by macrophages, including the tumour necrosis factor alpha (TNF- α), interleukin 1 (IL-1), interleukin 6 (IL-6), prostaglandin E2 (PGE2) and matrix metalloproteinase species (MMPs).³ In pathological conditions such as inflammation, TNF- α prevents macrophages from excreting intermediates such as TNF- α , IL-1, IL-6, PGE2, and MMPs.⁴ In pathological conditions such as inflammation, TNF- α

inhibits macrophage activity. The inhibition of collagen synthesis and the presence of MMPs increase collagen destruction. Collagen is absorbed continuously and replaced by inflammatory cells so that damage occurs in the periodontal tissue and the collagen density decreases.^{2,5} Untreated periodontitis can result in tooth loss. The goal of the treatment in periodontitis is to control bacteria as a local factor to minimise the systemic effects as a form of non-surgical treatment for periodontal disease.^{6,7} Host modulation therapy (HMT) is part of a periodontal therapy that is used as an adjunct to conventional periodontal treatments such as scaling and root planning, and aims to reduce damage and regenerate the periodontal tissue by reducing the destructive aspects of the host response.^{2,8}

The HMT properties can be found naturally in lemuru fish oil. Lemuru fish oil contains n-3 polyunsaturated fatty acids (PUFA), namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This has the potential as an anti-inflammatory that works by degrading potent eicosanoids in the form of PGE₂ and leukotriene B₄, so that the production of proinflammatory cytokines is inhibited. Lemuru fish oil will also affect growth factors by increasing the fibroblast growth factor (FGF), which plays a role in the proliferation of fibroblasts by stimulating collagen formation.^{9,10} Research on the topical application of catfish oil with content similar to EPA and DHA to tooth extraction sockets, in concentrations of 5% and 10%, showed an increase in the inactivation and amount of the bone morphogenetic protein-2 (BMP-2), which was most effective at a concentration of 10%. However, in the available research on lemuru fish oil with topical administration, there are no references proving which concentration is effective in curing periodontitis. Available research investigated the effect of applying lemuru fish oil with modified concentrations of 10%, 20% and 40%.¹¹ Given these limitations in the current research, the aim of this paper is to investigate the effect of lemuru fish oil gel on collagen density and on the width of the ligament in the periodontal tissue of Wistar rats induced by *P. gingivalis*.

MATERIALS AND METHODS

This research received the approval of the Ethics Commission of the Faculty of Dentistry, Hang Tuah University, with the number S.ket/068/KEPK-FKGUHT/XII/2019. The type of research was a true experimental laboratory investigation with a post-test only design. The samples used in this study were male Wistar rats (n = 30) aged 3–4 months, weighing 250–300g and divided into five groups, namely normal (K-) group, *P. gingivalis* bacterial induction without therapy (K+) group, *P. gingivalis* bacterial induction with 10% lemuru fish oil gel therapy (P1) group, *P. gingivalis* induction with 20% lemuru fish oil gel treatment (P2) group and *P. gingivalis* induction with 40% lemuru fish oil gel treatment (P3) group.¹¹

Periodontitis was obtained by bacterial induction using *P. gingivalis* (Pg) American Type Culture Cell (ATCC) 33277. The application of 2 ml of a 1 10⁹ CFU/ml solution of *P. gingivalis* bacteria, with 1.5 ml of a 1 10⁹ CFU/ml solution of live bacteria in phosphate buffered saline (PBS) with 2% of carboxymethylcellulose was carried out orally. In addition, 0.5 ml of bacteria was smeared using a cotton swab along the gingival groove on all teeth and anus in the colorectal area. The frequency of the *P. gingivalis* induction was three times in four days (0h, 48h and 96h). Periodontal tissue damage takes as long as four weeks counting from when the first bacterial induction is given.¹² Rats with periodontitis presented bleeding, redder than normal gingiva and a decrease in bone height, according to criteria described in a previous study.⁴

Lemuru fish oil was obtained from a caning waste factory from Banyuwangi, East Java (CV. Biji Sesawi, Banyuwangi, Indonesia). The induction of *P. gingivalis* was carried out first, then lemuru fish oil gel with concentrations of 10%, 20% and 40% was applied topically using a micro brush, once a day for 14 days. Approximately 1 ml/day of lemuru fish oil gel was applied topically each day on the lower jaw gingival sulcus of the Wistar rats using a micro brush (Cotisen, China). Therapy was carried out for 14 days in order to follow the period of the angiogenesis process, the process of osteoblast formation and to overcome the operator error process.⁴

The rats were euthanised one day after administration of the therapy using ketamine (Sigma, Germany) for termination at a dose of 100 mg/kg body weight (BW) intraperitoneally. Diazepam at a dose of 5 mg/kg BW was administered by inhalation. The rat was put into a container containing ether, and once it was in a sedative state, its neck was dislocated. Then the whole mandible was taken, and the rat was buried. The mandible was transferred to disposable polypropylene tubes (GP, China) containing 10% formalin liquid. Then the mandible was decalcified using 10% formic acid and cut sagittally between the mandibular left molar and the jawbone in the interdental area of the posterior mandible of the *Rattus norvegicus*, which was then stained using Mayer's haematoxylin (Orsatech, Germany).

The histometric analysis was carried out using an Olympus CX-22 (Olympus, Germany) and the Optilab program (Miconos, Indonesia), with 400x magnification. Histometric was performed with modification methods from the Damaiyanti and Mulawarmanti experiment, where the sample slides were divided into five fields of view and scored as +0 (no collagen fibres found in the wound area), +1 (density of collagen fibres in the low wound area (25%)), +2 (density of collagen fibres in moderate wound area (50%)), +3 (density of collagen fibres in tightly wound areas (75%)) and +4 (the density of collagen in the wound area is very tight (100%)).^{13,14}

The periodontal ligament width was measured in the cross section using a 400x magnification microscope, the Olympus CX22. The measurements were carried out using a raster image program at three locations in one field of

view with a micrometre unit. The reading was carried out by two observers.¹⁵

The data obtained were analysed using descriptive statistics to obtain a description of the data distribution and ranking in relation to the dependent variable (collagen density and periodontal ligament width), before proceeding with the analysis test. The Kruskal–Wallis test was used to compare the collagen density data between groups and the one-way analysis of variance (ANOVA) parametric statistical test was performed to analyse the periodontal ligament width. All tests were carried out using statistical product and service solutions (SPSS) version 24 (IBM, Armonk, US).

RESULTS

The data obtained from the results of the study were tabulated and analysed using statistics to test hypotheses using SPSS version 24. Because the collagen density data were measured as interval scores, nonparametric analysis was used. The mode of collagen density is shown in Table 1.

Table 1. Collagen density score mode value in the experiments

Group	Number of Samples	Mode
K-	6	4
K+	6	1
P1	6	1
P2	6	4
P3	6	3

Based on Table 1 and Figure 1, the lowest collagen density mode was observed for the K+ and P1 groups and the highest for K- and P2. A nonparametric test was performed using the Kruskal–Wallis test with a significance level of $p < 0.05$. The result of the significance between groups was $p = 0.001$ ($p < 0.05$). This shows that there are significant differences in the negative group, positive group and treatment group. Subsequently, the Mann–Whitney analysis was carried out. Significantly different results were obtained between groups, except between K- and P2, P3, P2 and P3, and K+ and P1, where no significant results were obtained, with $p > 0.05$ (Table 2).

Based on the results of the histological examination and the statistical calculations, the results of the width of the periodontal ligament obtained between groups are shown in Table 3 and Figure 2. The histopathology of the periodontal ligament width shown in Figure 2 was measured in three areas, with every field of view slide stained with haematoxylin eosin. Table 3 shows that the highest periodontal ligament width measured from bone and tooth was observed in the control positive group ($K+ = 425.85\mu\text{m}$) and the narrowest periodontal ligament width was observed in the control negative group ($K- = 299.87\mu\text{m}$).

Table 2. Significance test between groups using the Mann–Whitney test

Groups	K+	P1	P2	P3
K-	.002*	0.004*	0.589	0.132
K+		0.589	0.004*	0.004*
P1			0.015*	0.026*
P2				0.485

*significant ($p < 0.05$)

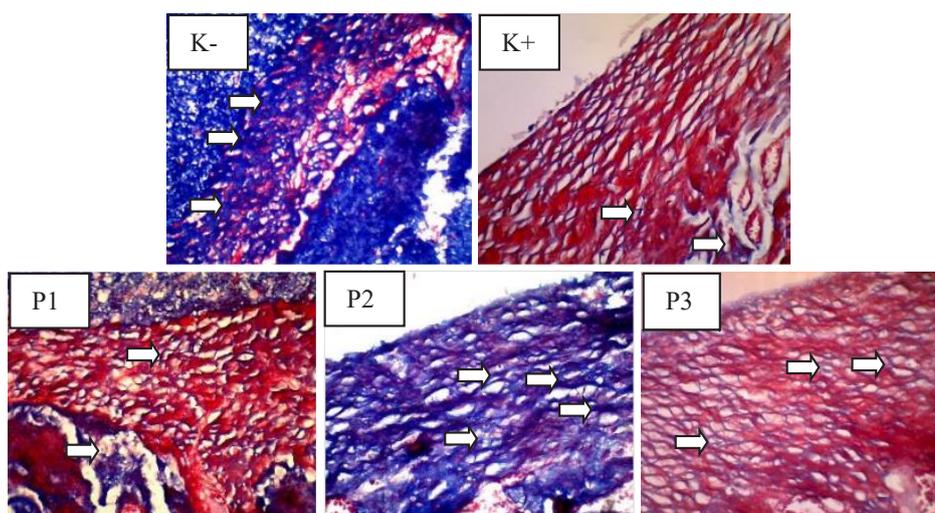


Figure 1. Histopathology of collagen density with MT staining at 400x magnification (collagen = blue colour) for the groups control negative (K-), control positive (K+), lemuru fish oil gel 10% (P1), lemuru fish oil gel 20% (P2) and lemuru fish oil gel 40% (P3).

The data collected on the periodontal ligament width were analysed using the Shapiro–Wilk normality test and resulted in a normal distribution because of $p > 0.05$. The one-way ANOVA parametric statistical test resulted in a significance of $p = 0.004$ ($p < 0.05$), which means that there is a significant difference in the periodontal ligament width. Subsequently, the post hoc Least Significant Differences (LSD) test was performed, as shown in Table 4, to determine the value of the difference between groups. The results of this test show that there are significant differences between the groups K- and K+, K- and P2, K- and P3, and K+ and P1.

The Spearman correlation analysis was used to determine the correlation between the parametric data, namely the width of the periodontal ligament, and the nonparametric data on collagen density scores. The correlation value obtained was -0.292 , which indicates an opposite relationship, i.e., the higher the collagen score, the smaller the periodontal ligament width. This shows that the higher the density value, the smaller the periodontal ligament widening, but the significance result obtained was $p = 0.11$, which means that the relationship between the collagen density and the periodontal ligament width is not significant ($p > 0.05$) as shown in Table 5.

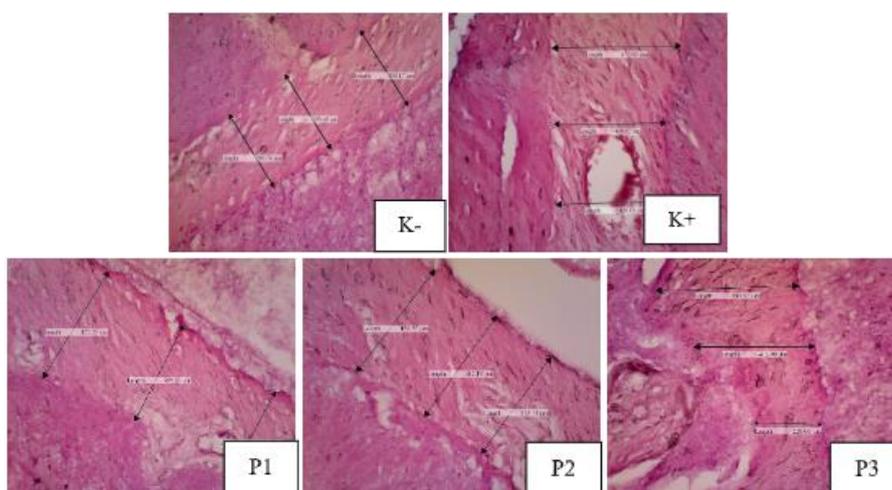


Figure 2. Image of the histopathological preparations showing the periodontal ligament width with haematoxylin eosin (HE) staining and 400x magnification (arrow between bone and tooth).

Table 3. Mean and standard deviations of the estimation of the periodontal ligament width at each group experiment

Groups	Replication	Average (μm)	Standard Deviation
K-	6	299.612	51.82
K+	6	425.850	61.54
P1	6	346.932	33.53
P2	6	370.159	49.42
P3	6	379.671	49.26

Table 4. Significance test of the periodontal ligament width

	K+	P1	P2	P3
K-	.000*	0.113	0.022*	0.01*
K+		0.011*	0.065	0.122
P1			0.428	0.267
P2				0.744

Table 5. Correlation between collagen density and periodontal ligament width

Correlations Spearman's rho		
Periodontal ligament width	Correlation coefficient	Collagen density score
	Sig. (2-tailed)	-0.292750482
	N	0.116427602
		30

DISCUSSION

In this study, the induction of *P. gingivalis* bacteria causes an inflammatory response in the periodontal tissue because they secrete biologically active endotoxins or LPS and cause the activation of macrophages. This plays an important role in the synthesis of pro-inflammatory cytokines such as IL-1 and TNF- α , PGE2 and hydrolytic enzymes.^{4,16}

Secretion of inflammatory mediators such as cytokines and prostaglandins responds to produce MMPs, proteolytic enzymes that affect the degradation of extracellular matrix macromolecules, namely collagen. In pathological conditions such as inflammation, TNF- α inhibits the activity of fibroblasts thereby inhibiting collagen synthesis as well as the presence of MMPs, which triggers collagen destruction.^{5,17} The induction of *P. gingivalis* bacteria was shown to cause periodontal ligament damage characterised by an increase of the periodontal ligament width, which is more visible in the positive control group compared to negative control group. According to the periodontal regeneration model conducted by Montevecchi *et al.*,¹⁸ a sign of periodontal ligament repair is a shorter width of the periodontal ligament compared to when the periodontal tissue damage occurred. This is because the formation of a good alveolar bone during the healing process causes the periodontal width to decrease.¹⁸

The groups induced by *P. gingivalis* bacteria and the group treated with 20% lemuru fish oil gel therapy had a significant difference in collagen density. This shows the positive effect of 20% lemuru fish oil gel on the increase of the collagen density. Eicosapentaenoic acid and DHA act as anti-inflammatory agents because of their ability to bind eicosanoids. They are contained in lemuru fish and compete with arachidonic acid, which can reduce the formation of MMPs and stimulating fibroblast cell regeneration. Fibroblasts, active inflammatory cells such as macrophages and neutrophils, epithelial cells and vascular endothelial cells stimulate the formation of MMPs. With the help of MMPs, fibroblasts digest the fibrin matrix and replace it with glycosaminoglycan. Over time, this extracellular matrix is replaced by type III collagen, which is also produced by fibroblasts. Furthermore, type III collagen is subsequently replaced by type I collagen during the maturation phase.

Lemuru fish oil also affect growth factors, namely FGF, which plays a role in the proliferation of fibroblasts so that it stimulates collagen formation. When extracellular matrix deposition occurs, collagen synthesis is augmented by growth factors, such as platelet-derived growth factor (PDGF), FGF and transforming growth factor beta (TGF- β), so the tissue remodelling process modulates the synthesis and the activation of metalloproteinases, an enzyme that functions to degrade the extracellular matrix. The result of the synthesis and degradation of the extracellular matrix is the remodelling of the connective tissue framework. This structure is the main feature of tissue healing in chronic inflammation.^{4,19}

The sample group with the induction of *P. gingivalis* bacteria and 10% lemuru fish oil gel did not result in a significant difference from the induced groups that did not receive therapy. This shows that lemuru fish oil gel with a concentration of 10% did not have a significant effect on collagen density and periodontal ligament-width. This is because the low doses were unable to stimulate growth factors such as FGF2. This mediator is needed in the healing process to trigger cell healing and differentiation and to initiate the recovery of damaged tissue.^{20,21}

Compared to the smaller concentration, the 20% concentration of lemuru fish oil gel showed better repair of the collagen density and the periodontal ligament. However, there was no significant difference in the results compared to the 40% concentration. This shows that if the gel preparation has a high concentration or excessive molecular weight, it will produce a thick gel layer when applied. The penetration of the gel through the hypodermic layer becomes less effective, thus making the therapeutic effect last longer.^{15,22}

The results showed that the negative control group did not have a significant difference in collagen density when compared with the study group treated with lemuru fish oil gel therapy at a concentration of 20%. Omega-3 fatty acids, especially EPA, have been shown to increase the number of fibroblasts and stimulate the formation of collagen. Eicosapentaenoic acid plays a role in increasing the amount of IL-6 cytokines, which consequently increases collagen production by fibroblasts and stimulates endothelial cells to form neovascular tissue through the process of angiogenesis and lead to the healing process.⁹

Lemuru fish oil contains unsaturated fatty acids consisting of omega-3 and omega-6. Omega-3 has been shown to regulate various proteins in periodontal tissue, such as MMP-8, MMP-13, MMP-14 and tissue inhibitor of metalloproteinases (TIMP). Omega-6 can affect the production of eicosanoids, PGE2, leukotriene and lipoxin. The ability of lemuru fish oil to influence eicosanoid metabolism is related to the long-chain structure of EPA and DHA, which has similarities to the long-chain structure of Arachidonic Acid (AA). Because of this, EPA and DHA can become AA competitor substrates to blend with the phospholipid membrane and directly inhibit the enzymes cyclooxygenase-2 (COX-2) and lipoxygenase. Inhibition of the cyclooxygenase pathway results in inhibited prostaglandins so that there is no monocyte activation mechanism to produce TNF- α and interleukin 1 β , which can inhibit collagen synthesis.^{9,23} The limitation in this study was the difficulty in ensuring that the whole 1ml of lemuru fish oil gel was fully absorbed in the sulcus of the periodontal ligament. Therefore, further research on the absorption capacity of lemuru oil is required, to ensure the effect of lemuru as a topical drug in the treatment of periodontitis.

Based on this research, lemuru fish oil gel influences the increase of collagen density in the periodontal tissue of Wistar rats induced by *P. gingivalis*. The concentration

of lemuru fish oil gel that had the most profound effect on the increase of the collagen density and the width of the ligament of the periodontal tissue of Wistar rats induced by *P. gingivalis* was 20%. The correlation between collagen density and the periodontal ligament width was negative and not significant.

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