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Research Report

Protection against periodontal destruction in diabetic condition with *Sardinella longiceps* fish oil: expression of matrix-metalloproteinase 8 and tissue inhibitor of metalloproteinase 1

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ABSTRACT

Background: *There is strong evidence to support the claim that periodontitis may be more prevalent among diabetic individuals. Collagen degradation represents one of the key events in periodontal destructive lesions. The level of matrix metalloproteinase 8 (MMP-8) and tissue inhibitor of metalloproteinase 1 (TIMP-1) are key to periodontal collagenolysis and associated with the severity of periodontal inflammation and disease. Host modulatory therapy has been proposed as a treatment for periodontal diseases. Sardinella longiceps (lemuru) fish oil containing polyunsaturated fatty acids (PUFAs), including omega 3 and 6, has been shown to possess therapeutic anti-inflammatory and protective properties effective against inflammatory diseases, including periodontitis.* ***Purpose:*** *The study aimed to examine the effect of dietary supplementation of Sardinella longiceps fish oil on protection against periodontal destruction resulting from the expression of MMP-8 and TIMP-1.* ***Methods:*** *Wistar rat samples are divided into four groups: a negative control group and three groups receiving Sardinella longiceps fish oil treatment (4 ml/ weight (Kg), 8 ml/ weight (Kg) and 16 ml/ weight (Kg). One week before treatment, all groups were administered with streptozotocin (STZ) 65 ml/ weight (Kg) and nicotinamide 110 ml/ weight (Kg) to induce diabetic conditions. Immunohistochemistry slides of periodontal tissues were prepared after three weeks of treatment. The expression of MMP-8 and TIMP-1 was counted using the HSCORE index, data was analyzed by means of non-parametric methods using Kruskal-Wallis, and Mann-Whitney tests.* ***Results:*** *Statistical analyses confirmed a significant increase in MMP-8 expression and a reduction in TIMP-1 expression in the negative control group compared to the treatment group ($p<0.05$). Meanwhile, the treatment group showed a significant reduction in MMP-8 expression and a marked increase in TIMP-1 expression, with the best result produced by the administering of 16 ml/ weight (Kg) Sardinella longiceps fish oil to the treatment group ($p<0.05$).* ***Conclusion:*** *Dietary supplementation of Sardinella longiceps fish oil can protect against periodontal destruction under diabetic conditions, by decreasing MMP-8 expression and increasing TIMP-1 expression.*

Keywords: *diabetes; lemuru; MMP-8 expression; periodontitis; TIMP-1 expression*

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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia, a high blood glucose level. From an epidemiological perspective, it is estimated that the prevalence of DM in Indonesia will reach 21.3 million people by 2030.[1] Hyperglycemia will trigger an increase in prostaglandin E2 (PGE2) and cytokine expression through activation of diacyl glycerol (DAG)-proteinkinase C (PKC). This increase in inflammatory mediators will, in turn, initiate a process of periodontal tissue destruction.[2]

Clinical studies have confirmed greater prevalence of periodontitis in patients with diabetes mellitus. Fibroblast cells do not function properly in environments featuring a high concentration of glucose. In addition, collagen produced by fibroblasts is susceptible to rapid degradation by matrix metalloproteinase (MMP) enzymes, the production of which is increased in diabetics rendering them susceptible

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to more rapid loss of attachment and bone damage.[3] The main MMPs found in diabetics include MMP-8 and MMP-9.[4] MMP-8 (collagenase 2) is one of the main biomarkers of connective tissue destruction in periodontitis.[4,5] The release of MMP-8 will be offset by that of the tissue inhibitor of metalloproteinase (TIMP). Periodontal damage will be affected by the ratio of MMP-8 to TIMP-1, the latter of which is produced by endothelial cells, and macrophages as protection against MMPs. An imbalance between MMPs and TIMP will lead to destruction during periodontitis pathology.[5,6] An in vitro study shows that the monocyte cells of diabetics experience an excessive response with increased expression of inflammatory mediators such as IL-1, TNFα and PGE2.[7] Inflammatory mediators such as IL-1β, and TNFα increased regulation of MMPs expression resulting in periodontal collagen degradation.[8]

Sardinella longiceps fish oil, obtained from fish canning industry waste, contains polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The EPA and DHA content of *Sardinella longiceps* fish oil is one of the highest.[9] Omega 3 contains several metabolites, resolvin and two-component protectins among others, that demonstrate the ability to reduce the production of pro-inflammatory enzymes and cytokine including COX2, TNFα and IL-β.[10] Administering omega-3 and omega-6 fatty acids together to research models whose feet had sustained injuries after diabetes induction resulted in more rapid wound healing. It seems that a combination of omega-3 and omega-6 can control cell diffusion and inflammation under diabetic conditions, the mechanism probably one of altering the fibroplastic or maturational phases of the healing response.[11] Based on the findings of research into omega 3 (EPA & DHA) diets in cases of periodontitis, it is known that omega 3 can reduce MMP, thereby inhibiting osteoclast activity.[12] This result was also produced by research into the apoptosis of osteoblasts in alveolar bone. The research involving the administering of a 1 ml/300 gram dose of *Sardinella longiceps* fish oil to rats indicated significantly smaller apoptosis osteoblasts in alveolar bone than those induced by lipopolysaccharide (LPS).[13] Provision of omega 3 for two weeks in the periodontitis model produced an increase in TIMP-1 levels.[14] Research involving the administering of omega 3 and omega 6 derived from Toman fish oil at doses of 4 ml, 8 ml and 16 ml/ weight (kg) once a day for seven days produced significant wound healing results. This was due to the ability of omega 3 and omega 6 to act as immunomodulators in controlling inflammation.

Based on the foregoing information, the researchers wanted to identify the effect of a diet containing *Sardinella longiceps* fish oil extract at doses of 4 ml, 8 ml, and 16 ml/ weight on the expression of MMP-8 and TIMP-1 in the periodontal tissue of Wistar rats as markers of periodontal destruction induced by diabetes mellitus.

MATERIALS AND METHODS

All experiments were approved by the Ethics Committee Faculty of dentistry, Universitas Hang Tuah with number certificate EC/013/KEPK-FKGUHT/VIII/2019 and performed following the guidelines. Experimental diabetes mellitus is produced by injecting a single dose of streptozotocin (Sigma Aldrich, Singapore). Forty male Wistar rats were injected with approximately 110 ml/ weight (Kg) of intra peritoneal nicotinamide (Merck, Germany) dissolved in phosphate buffer saline (PBS) (Merck Certipur, Germany). 15 minutes later a single intra-peritoneal dose 65 mg/ weight (Kg) of STZ was injected into Wistar rats that had fasted for between 8 and 12 hours overnight. After seven days of induction with STZ, the rats that presented an increased blood glucose level in excess of 126 mg/ ml were considered to be diabetic.[15,16]

After the induction of diabetes, the Wistar rats were divided into four groups, namely: K0 - diabetes induction, administered with *Sardinella longiceps* fish oil treatment; K1 - diabetes induction, administered with *Sardinella longiceps* fish oil treatment 4 ml/ weight (Kg); K2 - induction of diabetes, administered with *Sardinella longiceps* fish oil treatment 8 ml/ weight (Kg) and K3 - diabetes induction, administered with *Sardinella longiceps* fish oil treatment 16 ml/ weight (Kg). Treatment with *Sardinella longiceps* fish oil involved oral administration once a day for a period of three weeks.

Both the control rats and their medicated counterparts were sacrificed after three weeks' administration of *Sardinella longiceps* fish oil or one month post-diabetes induction with STZ. The rats were inserted in a glass tube and administered a lethal dose of ether for three minutes. Following removal of their jaws, which were subsequently immersed in a fixing solution, the rats were interred. The specimen processing technique was continued using a paraffin method, immunohistochemistry staining techniques with monoclonal primary antibodies, MMP-8 and TIMP-1 and secondary antibodies that had been biotinated.[17]

Calculation of MMP-8 and TIMP-1 expressions using a modification of HSCORE technique was conducted with an Olympus CX-21 microscope using an optilab program at 400x magnification through three visual fields. The intensity of scores was assessed according to category: 0, absent; 1, weak; 2, moderate and 3, intense before being included in the formula HScore = Pi (i +1) where i is the intensity score, Pi is the percentage of the number of cells stained and 1 represents a correction factor.[18] The data was analyzed by means of a non- parametric Kruskal-Wallis test the data from which was in ordinal form. In cases of difference, a Mann-Whitney test was subsequently conducted to identify the significance difference.

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RESULTS

Observation and calculation of MMP-8 and TIMP-1 expression in the periodontal tissue area were conducted one month after diabetes induction and treatment. The results indicated that the highest median value of MMP-8 occurred in the K0 group compared to the treatment group, while the highest median of TIMP-1 was observed in the K3 groups compared to the control group. It can be seen that the highest MMP-8 score was recorded in the K0 group and the lowest score in the K3 group (Figure 1).

The results of the TIMP-1 calculation show that the highest score occurred in the group K3 (Figure 2). Based on the contents of Table 1, there were significant differences between the control group and groups K1, K2 and K3 as indicated by the value $p<0.05$ for both the MMP-8 and TIMP-1 scores. In contrast, with regard to the Mann-Whitney test, there were no significant differences between MMP-8 scores of the control, K1 and K2 groups where the value of $p> 0.05$.

These results showed that K3 group registered the lowest MMP-8 score expression and the highest TIMP-1

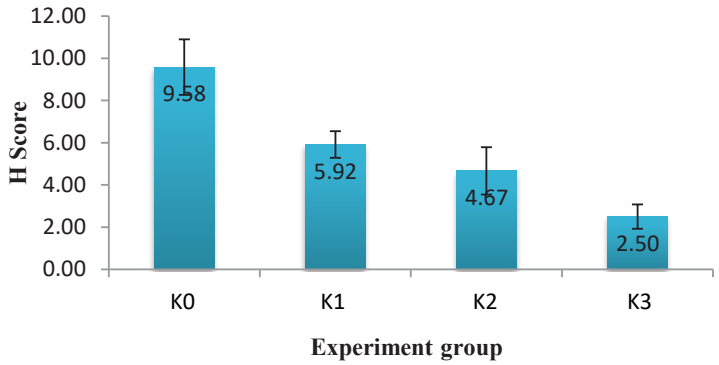

Figure 1. MMP-8 expression after a 3-week period of treatment with *Sardinella longiceps* fish oil.

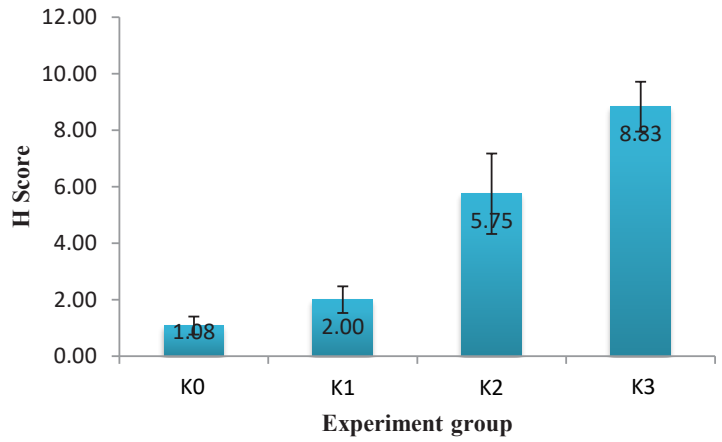

Figure 2. Expression of TIMP-1 after 3 weeks treatment with *Sardinella longiceps* fish oil.

Table 1. MMP-8 significance test results between groups

	Group K1		Group K2		Group K3	
	MMP-8	TIMP-1	MMP-8	TIMP-1	MMP	TIMP-1
Negative Control group	0.019	0.037	0.02	0.02	0.019	0.02
Group K1			0.078	0.02	0.019	0.02
Group K2					0.019	0.021

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score compared to the other groups, while the K0 group had the highest MMP-8 score and the lowest TIMP-1 score (Table 1). The expression of MMP-8 and TIMP-1 was calculated using the color intensity and proportion scores, which were then calculated using the formula. The dark brown IHC staining visible in the picture indicates a positive expression (Figure 3 dan 4).

DISCUSSION

Systemic conditions have been identified as the cause of an increase in periodontal disease. Biological mechanisms known to occur under diabetic conditions include: increased production of advanced glycation end products (AGEs), hyperinflammatory reactions, poor quality collagen,

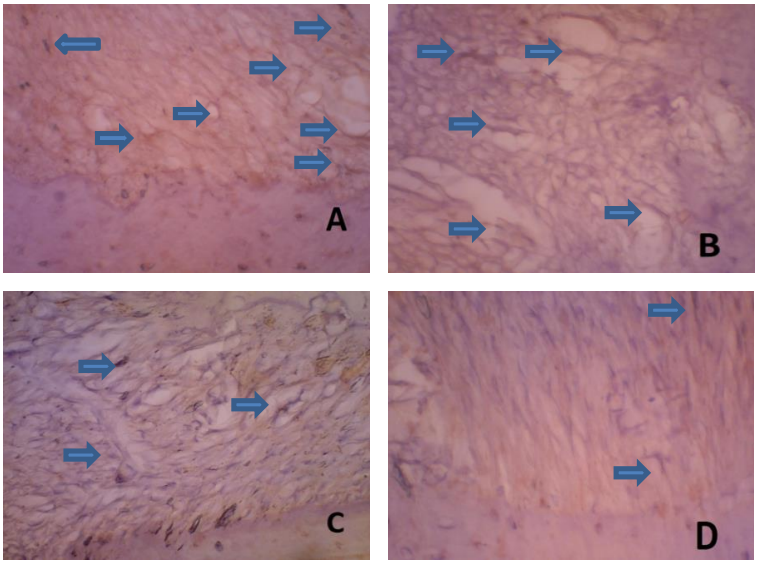

Figure 3. MMP-8 expression (dark brown, blue arrow) in periodontal ligament at 400x magnification. (A) Group K0 (Diabetes induction without treatment); (B) group K1 (Diabetes induction, *Sardinella longiceps* oil treatment 4 ml/ weight (Kg)); (C) group K2 (Diabetes induction, *Sardinella longiceps* fish oil treatment 8 ml/ weight (Kg)); (D) group K3 (Diabetes induction, *Sardinella longiceps* fish oil treatment 16 ml/ weight (Kg)).

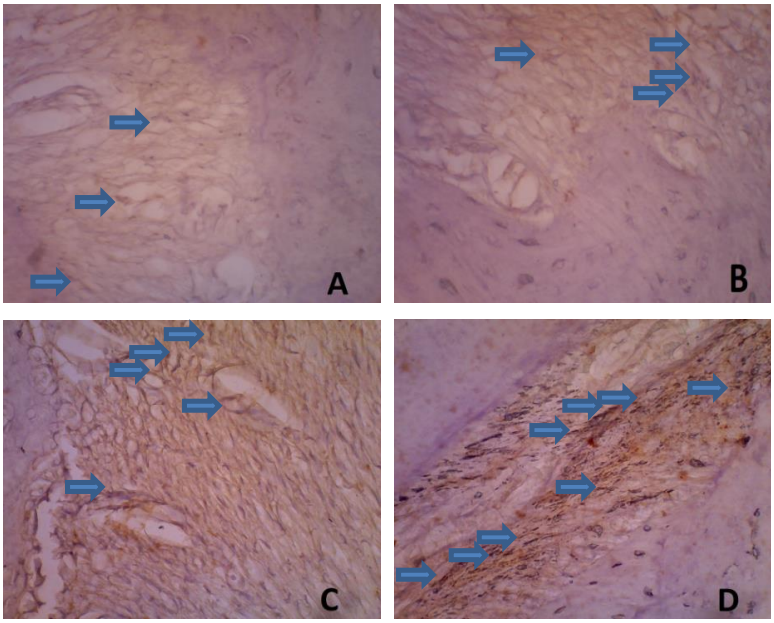

Figure 4. TIMP-1 expression (dark brown, blue arrow) in periodontal ligament at 400x magnification. (A) Group K0 (Diabetes induction without treatment); (B) group K1 (Diabetes induction, *Sardinella longiceps* oil treatment 4 ml/ weight (Kg)); (C) group K2 (Diabetes induction, *Sardinella longiceps* fish oil treatment 8 ml /weight (Kg)); (D) group K3 (Diabetes induction, *Sardinella longiceps* fish oil treatment 16 ml/ weight (Kg)).

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changes in microcirculation, host defense dysfunction and increased matrix metalloproteinases. The formation of AGEs plays an important role in increasing the sensitivity of the formation of inflammatory mediators by endothelial cells and monocytes. The accumulation of AGEs in the plasma and tissues in diabetic patients is associated with the occurrence of periodontal disease.[19,20]

The pathology of activation or increase in MMPs and the production of TIMP protection systems by bacteria or by inflammatory mediators is characteristic of chronic periodontitis.[5] This characteristic was shown by the STZ-induced negative control group for diabetes after one month and subsequently examined using IHC, which resulted in a small increase in MMP-8 and TIMP-1 production (Table 1), collagen degeneration and characteristic of chronic periodontitis. Levels of MMPs and TIMP-1 are very important because they can indicate the progression of periodontitis. Increased MMP and production of TIMPs are less able to direct periodontal attachment loss.[5,14]

The administration of *Sardinella longiceps* fish oil was conducted one week post-induction for three weeks, while the research subjects were euthanised one month post-STZ induction. The results of this study indicated the smallest MMP-8 expression to have occurred in the 16 ml/ weight (Kg) *Sardinella longiceps* fish oil group and demonstrated a significant difference compared to the control group, 4 ml/ weight (Kg) *Sardinella longiceps* fish oil group and 8 ml/ weight (Kg) *Sardinella longiceps* fish oil group. The decrease in MMP-8 expression was in accordance with the results of TIMP-1 expression in the 16 ml/ weight (Kg) *Sardinella longiceps* fish oil group which experienced a significant increase compared to the control group, 4 ml/ weight (Kg) *Sardinella longiceps* fish oil group and 8ml weight (Kg) *Sardinella longiceps* oil group. It was concluded that the 16 ml/ weight (Kg) lemuru fish oil group had the lowest MMP-8 expression and the highest TIMP-1 expression.

The control group results indicated an increase in MMP-8 without being followed by a rise in TIMP-1. In theory, this could lead to the degradation of collagen and destruction of periodontal tissue. This study proves the expression of MMP-8 in the negative control group, diabetic rats increased without being offset by the production of MMP-8 inhibitors, namely TIMP-1, which is limited in scale. Increased MMP-8 has been reported to be associated with the development of gingivitis into periodontitis.[5]

The treatment group showed results that support the theory that a decrease in MMP-8 and an increase in TIMP-1, due to the administration of *Sardinella longiceps* fish oil containing omega 3, can reduce the production of inflammatory mediators such as PGE2. Prostaglandin E2 (PGE2) causes stimulation of inflammatory mediators and MMPs, including osteoclast formation.[8] Inhibition of PGE2 production can, in turn, lead to restrictions on MMP production, while *Sardinella longiceps* oil with polyunsaturated acid (PUFA) content can reduce PGE2 production.[10] The positive effects of omega-6 have also been reported in terms of their ability to affect the production of eicosanoids, PGE2, leukotrienes and lipoxins. They also inhibite cyclooxygenase, promote growth hormone secretion from the anterior lobe of the pituitary gland by derived eicosanoids from omega-6 fatty acids. Moreover, they prevent a reduction in arachidonic acids in plasma, as well as membrane phospholipids in diabetes, mitogenic effects and an increase in collagen formation.[11]

Sardinella longiceps fish oil contains PUFA consisting of omega 3 and 6. Omega 3 is proven to regulate various proteins in periodontal tissues such as MMP-8, MMP-13, MMP-14 and TIMP-1 in the periodontitis-induced LPS model.[14] In experimental studies of periodontitis in rats, the use of selective COX-2 and prophylactic omega 3 fatty acids, both singly and in combination, caused inhibition of MMP-8 expression in gingival tissue.[8] This finding is consistent with the results of the study of the *Sardinella longiceps* oil treatment group which experienced a decrease in MMP-8 expression compared to the negative control group. The largest reduction occurred in the 16 ml/ weight (Kg) *Sardinella longiceps* fish oil treatment group.

TIMPs that control MMP activity and act as regulators of extracellular matrix destruction by MMP have an important role to play in tissue remodeling and pathology from periodontal tissue destruction. TIMP levels are generally more elevated in healthy periodontal tissues than those affected by periodontal inflammation, which leads to the excessive production of MMPs.[8,5] This finding is in accordance with the study of the control group which showed high MMP-8 expression, while that of TIMP-1 was low. Contrastingly, in the treatment group MMP-8 expression decreased and TIMP expression increased. In conclusion, administering *Sardinella longiceps* fish oil to diabetic rats was found to reduce MMP-8 expression and increase TIMP-1 expression. The most impressive results occurred in the group which received a 16 ml/ weight (Kg) dose of *Sardinella longiceps* fish oil.

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