Interleukin-17a and Tumor Necrosis Factor Receptor-Associated Factor-6 Expressions on Administration of Nannochloropsis Oculata During Orthodontic Relapse

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Abstract

Orthodontic relapse is the return of the tooth position, after orthodontic treatment, to the position of the last tooth at the end of treatment. Nannochloropsis oculata is one of the best marine products in Indonesia. It contains natural ingredients and various active ingredients such as EPA DHA, oxylipin, triterpene, vitamins, minerals, polysaccharides, flavonoids, and alkaloids, which can prevent an orthodontic relapse. This study aimed to determine the effect of 3% of Nannochloropsis oculata on Interleukin-17A (IL-17A) tumor necrosis factor (TNF) receptor-associated factor (TRAF-6) expressions during the orthodontic relapse. The research was conducted with a Post-Test Only Group design. Twenty-four male Cavia Cobaya were divided into three groups: K(-) as a negative control group, K(+) as a positive control group which was given orthodontic relapse, and P group which was given orthodontic mechanical pressure to get a relapse and 3% of Nannochloropsis oculata. The IL-17Aa and TRAF-6 expressions were examined with immunohistochemistry. The data were evaluated using One-way ANOVA and Tukey tests ($p < 0.05$).

The results of this study proved that the addition of 3% Nannochloropsis gel reduced TRAF-6 expression in the Nanno group compared to the relapse group; and it increased the IL-17A expression in the Nanno group compared to the relapse group.

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Introduction

Relapse occurs after orthodontic treatment. Relapse is a change in the position of the tooth back to the original position before moved by orthodontic appliances.[1] Immediately after the orthodontic pressure is released, the tooth becomes unstable due to the reorganization of the gingival fibers and the periodontal ligament fibers influenced by an increase in mechanical stress.[2] This stress is influenced by the trans-septal fibers; during teeth growth, especially mandibular growth, the condition of occlusal stability has not been achieved and the soft tissue pressure around the teeth muscles as well as induces alveolar bone resorption by osteoclasts.[3,4] Due to this resorption, patients have to use retainers for a minimum of six months for retaining the balance and stability of the teeth.[5] The 40% chance of a relapse may occur rapidly up to about 4-6 months due to the movement of the previous teeth. In addition, it can also last up to 1 year after the orthodontic appliance is removed. Tooth relapse is still a problem that many clinicians encounter as it can hinder the success of orthodontic treatment. It is a physiological response of the periodontium to the orthodontic pressure received.[6,7]

Orthodontic mechanical stress causes strain that affects periodontal ligament vascularization and blood flow resulting in local synthesis and release of neurotransmitter molecules, cytokines including IL-17A, growth factors, colony-stimulating factors, and arachidonic acid metabolites.[8,9] Reorganization of the periodontal ligament after orthodontic tooth movement is very important to prevent the tooth from relapsing to its original position. The reorganization of the periodontal ligament is important for stability because the periodontium contributes to control tooth position.[10]

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Interleukin 17 (IL-17A) is originally an antigen-8-linked cytotoxic T-lymphocyte, an inflammatory cytokine produced exclusively by activated T-cells (Th 17 cells). IL-17A is an important mediator of autoimmune diseases including rheumatoid arthritis, multiple sclerosis, and allergic respiratory infections.[12] In the bones, IL-17A can stimulate RANKL on osteoblasts and support the expression of RANK, the receptor for RANKL on the surface of osteoclasts. Th17 cells can express RANKL but cannot activate osteoclasts through RANK-RANKL interaction.[13] IL-17A is also known to induce RANKL production by osteoblasts in periodontitis.[12] The secretion of IL-17A by Th 17 cells and the induction of RANKL in osteoblasts can lead to activate osteoclasts in bone resorption.[13] The large orthodontic pressure could induce IL-17A expression in the tooth roots of rats that were adsorbed on day 7. During the orthodontic tooth movement, the secretions of sRANKL and OPG from periodontal ligament cells were stimulated by IL-17A. The release of sRANKL increased according to the level of IL-17A and the release time, while the level of OPG decreased.[12,13]

Tartrate Resistance Acid Phosphatase 6 (TRAP 6) is a useful enzyme for osteoclast biomarkers, commonly used as a method to observe the distribution of osteoclast cells on tooth movement. Osteoclasts secrete large amounts of TRAP in the ruffled border, lysosomes, Golgi cisternae, and vesicles.[14,15] Osteoclasts undergo a serial differentiation stage to become resorptive mature cells. The intracellular signaling cascade of osteoclast-specific gene transcription regulation is activated by M-CSF and RANKL, and during the differentiation process, osteoclast precursor cells express RANK which is the receptor for RANKL. The differentiation process occurs when the signal is transferred from RANKL through the transcription factors, AP-1 and NFκB. Several proinflammatory cytokines including TNF-, IL-6, and IL-1 together enhance osteoclast genesis. TNF-α signaling occurs via NF-κB and MAPK, while IL-6 flows through the JAK-STAT pathway. Activation of osteoclasts with an inflammatory mechanism causes resorption with the release of bone resorption markers, one of which is TRAP.[16-18]

Nannochloropsis oculata is a microalga with promising biological resources to be developed and applied as a new product. It has a high source of EPA and DHA, oxylipin, and turpentine, alkaloids, and flavonoids. Terpenoids function to stimulate the formation of collagen.[19] Alkaloids may affect the formation of collagen mediated by platelet aggregation. [11] Flavonoids function to limit the release of inflammatory mediators. The anti-inflammatory activity of flavonoids is carried out by inhibiting cyclooxygenase and lipoxygenase to limit the number of inflammatory cells that migrates to the tissue. If the inflammatory reaction is shorter, proliferation will occur immediately.[19,20] This current study was conducted in vivo, which is research administering Nannochloropsis oculata extracts systemically to a body of living things to determine the effectiveness of the extracts. It investigated whether Nannochloropsis influenced orthodontic relapse through the expression of TRAF-6 as an osteoclast marker and IL-17A cytokine.

Materials and methods

This study was a true experimental laboratory study using a completely randomized control-group post-test design. Ethical permission for this study was obtained from the Ethics and Scientific Research Committee of Experimental Animal Use in the Faculty of Dental Health of Hang Tuah University. This study included laboratory experimental research. It involved calculations of sample size and possible number of experimental animals dying. It then resulted in 8 Cavia Cobaya as a minimum number of samples in each group. The samples were selected using simple random sampling, and they were randomly divided into three groups: (K -) negative control group (no treatment was given), (K +) positive control group (orthodontic relapse was given) and (P) treatment group (Orthodontic relapse + 3% of Nannochloropsis oculata gel were given).

When applying orthodontic appliances, an incisive band was glued together using GIC (Glass Ionomer Cement) and helical springs on the maxillary central incisors of Cavia Cobaya. The appliances were activated using coil pliers between the maxillary incisors with a pressure of 0.62 N on Cavia Cobaya (equivalent to 20 N in humans) for 14 days and were removed for 7 days for orthodontic relapse.

Nannochloropsis oculata extract is obtained from the Situbondo Research Center for

Marine Aquaculture (Situbondo Balai Budidaya Air Payau) using the whole extract method in which 0.1 mg of Nannochloropsis oculata gel with a concentration of 3% in phosphate buffer saline (PBS) was administered to the gingival sulcus once a day. Administration of the extracts to the gingival sulcus with a blunt insulin syringe was done every day for 21 days. The gel dose given to Cavia Cobaya was 0.025 ml once a day. The gel was applied topically to the gingival sulcus of Cavia Cobaya in the area of tension.

On the 22nd day, the three groups were sacrificed by anesthetizing with ketamine-xylazine and decapitating them. Then, the maxillary of Cavia Cobaya and their teeth were taken. Network fixation was done to them with formalin buffer solution. Then, they were cut and stained by the immunohistochemical method to examine the expression parameters of TRAF-6 and IL-17Aa in the pressure area of periodontal ligament viewed through a light microscope with a magnification of 1.000x. The results were analyzed using the ANOVA statistical analysis followed by multiple comparisons to determine differences between groups using the Tukey HSD test.

Results

This study involved two variables. The first one was observation and examination results of the expression of Tartrate Resistance Acid Phosphatase 6 (TRAF 6) using the immunohistochemical method and specific antibodies against TRAF - 6 (monoclonal anti-TRAF-6). Expression of TRAF-6 was the enzyme secretion by osteoclast cells, which played a role in the bone resorption process, and it gave a positive reaction to anti-TRAF-6 monoclonal antibodies characterized by the presence of brown color with 1,000x magnification as shown in Figure 1.

The second variable in this study was observation and examination results of the expression of Interleukin 17 (IL-17A) using the immunohistochemical method and specific antibodies against IL-17 (monoclonal anti-IL-17A). Expression of IL-17A is the production of inflammatory cytokine by macrophage cells, which gave a positive reaction to anti-IL-17A monoclonal antibodies characterized by the presence of brown color in the area of attraction for tooth movement with 1,000x magnification as shown in Figure 2. The mean value and standard deviation of TRAF-6 and IL-17A expressions in the area of attraction in each study group shown in Table 1. From the results of the homogeneity test in Table 2, the significance values of the TRAF-6 and IL-17A expressions were more than 0.05 ($p > 0.05$), so it can be concluded that the research data are homogeneous. From the results of the normality test in Table 3, the significance values of Shapiro Wilk on the TRAF-6 and IL-17A expressions were more than 0.05 ($p > 0.05$). It can be concluded that the research data were normally distributed. The data were then analyzed using the One-way ANOVA test to determine the differences in TRAF-6 and IL-17 expressions (Table 4). The examination obtained significance values of 0.000 both in TRAF-6 and IL-17A expressions ($p < 0.05$). This indicates a significant difference in the expressions between the negative group and the positive group with the treatment group. Based on this, the data were continued to proceed with the Tukey HSD test to find out which groups had differences (Table 5).

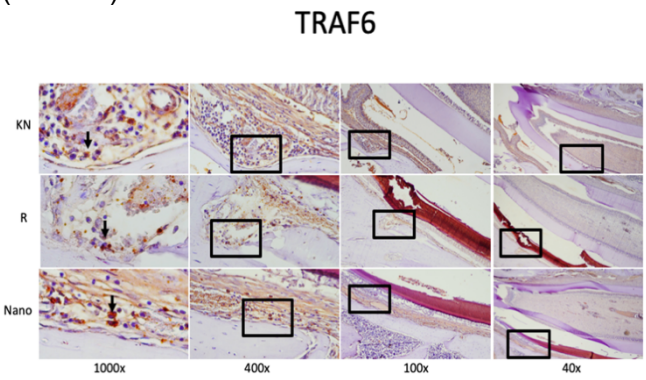

Figure 1 .The positive TRAF-6 expression in the relapse treatment group than in the Nano group. The negative control group, however, gave a negative reaction, and it did not give a color reaction.

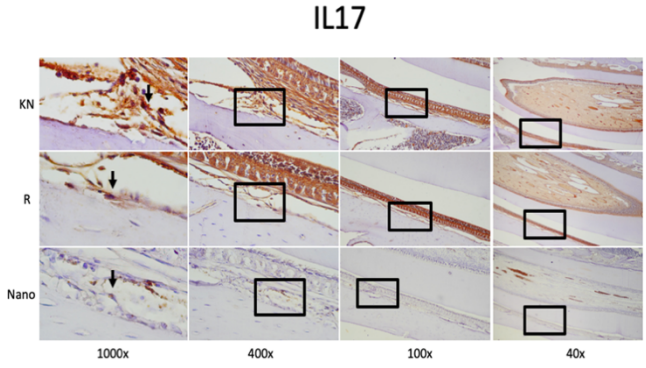

Figure 2. brown color positive IL-17A expression

in the Nano treatment group than in the relapse group. While the negative control group gave a negative reaction and did not give a color reaction.

Groups	n	PARAMETERS			
		TRAF - 6		IL- 17A	
		Mean ± SD	Min-Max	Mean ± SD	Min-Max
Normal	8	3.30 ± 1.160	2 - 5	3.20 ± 1.687	1 - 6
Relapse	8	12.10 ± 1.912	10 – 15	4.50 ± 1.509	2 - 7
Nanno	8	3.90 ± 1.449	2 - 6	15.00 ± 2.625	12 - 19

Table 1. The mean and standard deviation (SD) of TRAF-6 and IL-17A expressions.

Homogeneity Test of Variances

	Levene Statistics	df1	df2	Sig.
TRAF-6	1.563	2	27	.228
IL-17A	2.996	2	27	.067

Table 2. Levene's test results.

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
Group		Statistic	df	Sig.	Statistic	df	Sig.
TRAF-6	K-	.202	10	.200*	.878	10	.124
	K+	.221	10	.182	.860	10	.076
	nano	.333	10	.002	.828	10	.032
IL-17A	K-	.262	10	.051	.894	10	.188
	K+	.230	10	.144	.947	10	.638
	nano	.177	10	.200*	.900	10	.219

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 3. Shapiro Wilk test results.

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TRAF-6	Between Groups	483.467	2	241.733	102.141	.000
	Within Groups	63.900	27	2.367		
	Total	547.367	29			
IL-17A	Between Groups	837.267	2	418.633	104.562	.000
	Within Groups	108.100	27	4.004		
	Total	945.367	29			

Table 4. One-way ANOVA Test results.

Multiple Comparisons

Tukey HSD

Dependent Variables			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
TRAF-6	K-	K+	-8,800*	.688	.000	-10.51	-7.09
		nano	-.600	.688	.662	-2.31	1.11
	K+	K-	8,800*	.688	.000	7.09	10.51
		nano	8,200*	.688	.000	6.49	9.91
	nano	K-	.600	.688	.662	-1.11	2.31
		K+	-8,200*	.688	.000	-9.91	-6.49
IL-17A	K-	K+	-1.300	.895	.329	-3.52	.92
		nano	-11,800*	.895	.000	-14.02	-9.58
	K+	K-	1.300	.895	.329	-.92	3.52
		nano	-10,500*	.895	.000	-12.72	-8.28
	nano	K-	11,800*	.895	.000	9.58	14.02
		K+	10,500*	.895	.000	8.28	12.72

*. The mean difference is significant if less than 0.05.

Table 5. Tukey HSD results.

This test is a follow-up test that is used after it is known that there are significant differences in the One-way ANOVA test results of TRAF-6 and IL-17A expressions. The HSD test was performed to find any significant differences in the number of blood vessels between groups. The two treatments are significantly different if the significant values of the HSD test results are less than the research error rate of 0.05 (5%). Conversely, if the significant values of the HSD test results are greater than 0.05 (5%), then the two treatments are not significantly different. According to the TRAF-6 expression data, the TRAF-6 expression in positive control group was significantly different from that in the negative control group and the treatment group. Meanwhile, the negative control group was not significantly different from the treatment group. In addition, the IL-17A expression in the negative control group was significantly different from that in the treatment group; the positive control group was significantly different from the treatment group but not significantly different from the negative control group.

Discussion

Orthodontic relapse is the return of the tooth position, after orthodontic treatment, to the position of the last treated tooth. The position of the teeth could return to their original tooth position because of some factors such as periodontal tissue remodeling, appliance pressure and so on. Nannochloropsis oculata is one of the best marine products in Indonesia which contains natural ingredients and various active ingredients such as EPA DHA, oxylipin,

triterpene, vitamins, minerals, polysaccharides, flavonoids, and alkaloid. It is considered able to prevent orthodontic relapse. To determine the effect of Nannochloropsis oculata, this current study had observered and examined IL-17Aa and TRAF-6 expressions during orthodontic relapse.

It has been reported that compression of the complex PDL vascular causes ischemia resulting in tissue necrosis. Thus, the presence of immune cells in an environment devoid of bacteria is likely related to the presence of damaged host cells and the need to recruit inflammatory cells. IL-17 is part of relatively recently identified cytokine groups that play a key role in bone remodeling and mediate switches in cell-mediated immune responses. Upregulated IL-17 induces local inflammation, produces inflammatory and osteoclastogenic mediators, such as tumor necrosis factor-a, and promotes the expression of nuclear factor ligand-receptor activator kB (RANKL). It has recently been shown that IL-17 upregulates IL-6 and MMP-1 expression in human periodontal ligament cells, leading to collagen degradation associated with orthodontic tooth movement.[21]

Although several biochemical cascades regulate bone homeostasis, one of the most dominant pathways involves the interleukin 1 (IL-1) cytokine family as signal transducers. Interleukin17 (IL-17) is a T cell-derived cytokine that plays a role in osteoclast development. Studies have shown that IL-17 is expressed in the early stages of the immune response and acts on osteoblasts resulting in the expression of osteoclast differentiation factor, also known as RANKL. This factor induces osteoclast progenitors to differentiate into mature osteoclasts. IL-17 promotes the production of cytokines and chemokines to attract neutrophils and macrophages to move to sites of inflammation. The IL-17 receptor is commonly expressed in fibroblasts and epithelial cells, and innate immune cells, such as macrophages and neutrophils.[22] Takahashi *et al.* (2005) reviewed the IL-17 receptor's role in the immunopathology of periodontal disease and concluded that IL-17 is produced locally at the site of periodontal lesions from activated gingival T-cells. In periodontal lesions, IL-17 induces the production of IL-6, RANK, and RANKL. All of these factors are cytokines that promote bone resorption.[23]

In addition to bone resorption, IL-17 also plays a role in bone formation. IL-17 is a growth factor for mesenchymal stem cells by stimulating the formation of colony-forming units-fibroblasts in marrow stromal cells. In another study, IL-17 was shown to switch the differentiation of the C2C12 (a myoblast cell line) into the osteoblastic pathway. Both studies concluded that IL-17 is capable of directing the differentiation of mesenchymal stem cells into osteogenic lineages.[24,25]

Previous studies have shown that compressive forces increase the production of IL-17 and its receptors by osteoblast-like cells, influencing osteoclast genesis. In addition, IL-17A has been shown to induce cancer cell invasion through up-regulating MMP-2 and MMP-9 expressions.[26] A previous study also found that IL-17 expression was positively correlated with MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, and MMP-13. Therefore, previous researchers hypothesized that IL-17 could promote MMP expression during OTM.[27] Moreover, IL-17 regulates the RANKL signaling pathway by inducing up-regulation of the RANK receptor expressed by osteoclast precursor cells. It is confirmed that IL-17 promotes osteoclast differentiation via RANKL signaling pathway from fibroblast-like synoviocytes.[28]

The TRAF-6 signaling pathway plays an important role in the RANK-mediated stage of osteoclast activation. Although the signaling pathways for cell differentiation and osteoclast activation are unclear, M-CSF and RANKL produced by matrix cells are consistently important factors produced by osteoclasts. After RANKL integrates with osteoclasts and RANK, TRAF-6 in the endochylema integrates certain structural domains of RANK. There are five classic TRAF-6 pathways: the NF-B pathway, the MAPK pathway (JNK iter, p38MAPK pathway, and EPK pathway), and the Src pathway.[29]

TRAF, more importantly TRAF6, is key to understanding how the RANK ligand (RANKL) links cytoplasmic signaling to the nuclear transcriptional program. Tang et al. reported that the expression level of TRAF6 in hPDLC is markedly upregulated upon activation of Toll-like receptors and nucleotide-binding oligomerization domains. Meanwhile, this research group also found that TRAF6 was a major signaling molecule for IL-17- hPDLC-dependent regulation of RANKL. TRAF-6 interference inhibits IκBα and JNK phosphorylation and suppresses IL-17-mediated RANKL expression, thereby regulating

inflammatory bone destruction.[30] A previous study investigated whether TRAF6 was required for TNF-induced osteoclast formation using osteoclast precursors on TRAF6-deficient mice. The results showed that TNF- could not induce osteoclast formation and suggested that TRAF6 was required for in vitro TNF-induced osteoclast formation. However, TRAF6 is not a common adapter protein for TNFR. While TNF- synergistically enhances RANKL-induced osteoclast formation.[31]

Furthermore, osteoclast differentiation demands the presence of TNF family receptors, for example, RANK, which specifically exacerbates biochemical signaling through intracellular recruitment of TNF receptor-associated factors (TRAFs). The recruitment follows the ligand binding and receptor oligomerization. Also, TNF-associated apoptosis-inducing ligand (TRAIL) is associated with the induction of osteoclast differentiation via a TRAF6-dependent signaling pathway, leading to consequent inhibition of RANK-ligand-induced osteoclast differentiation (RANKL). Besides that, TRAF6, as an important adapter molecule, is involved in downstream RANKL/RANK, TLR, IL-1βR, and other classical pathways. It is a key factor for activating the NF-kB pathway to promote the release of proinflammatory cytokines and osteoclast-associated factors. It was reported that the suppression of TRAF6 inhibited p38 phosphorylation to decrease proinflammatory cytokine expression.[32]

Conclusions

The analysis results in this study showed that the addition of 3% of Nannochloropsis gel reduced TRAF-6 expression in the Nanno group compared to the relapse group. It also increased IL-17A expression in the Nanno group compared to the relapse group.

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Declaration of Interest

The authors report no conflict of interest.

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