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Interleukin-1α And Alkaline Phosphatase Gene Expression Towards Osteoblast Cell Culture Post Alpha-Mangostin Exposure (An In-Vitro Laboratory Experimental Study)

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ABSTRACT

Currently in the Dentistry field, there are lots of things that are related to the process of bone regeneration. Bone healing deeply relies on the early inflammation phase, which is affected by local and systemic responses towards adverse stimuli. Indonesia has lots of traditional plants that can be used as an alternative for complementary therapy and alternative medicine. The natural substance alpha-mangostin was utilized in this study because earlier research suggested that it has anti-inflammatory effects via cytokine expression and increases osteoblastic differentiation by assessment of multiple osteogenic markers following LPS administration. This study uses a post-test-only control group design to look at the expression of IL-1 and ALP genes in osteoblast cell culture using Real-time PCR. This study was divided into four experimental groups, one control group and three treatment groups. IL-1α gene expression increased with LPS induction (E1), and decreased in a group with α-mangostin (E2) and group with LPS and α-mangostin (E3). Whereas ALP expression decreased in a group with LPS (E1) and group α-mangostin (E2) and remained constant with the control group in the group given LPS and α-mangostin (E3). From this study, it can be concluded that LPS can induce inflammation in osteoblast cell cultures and α-mangostin can reduce inflammation which is characterized by decreased expression of the IL-1 gene, but has not shown any effect on osteogenesis.

Keywords: ALP; IL-1α; inflammation; human and health; osteogenesis; osteoblast

INTRODUCTION

The bone regeneration process after tooth extraction on a socket is comparable to the general healing process, however, it involves both bone and soft tissues.[1] The inflammatory phase at the

beginning of bone injury plays an important role in the bone healing process, which is affected by local and systemic responses towards adverse stimuli. Continuous inhibition of the inflammation process caused by adverse stimuli could lead to bone healing disruption. The inflammation process is continued with the bone formation process through osteogenesis. Wntsignaling pathway is a pathway for osteogenesis through inflammation by RUNX-2 formation which stimulates other osteogenesis markers, such as lain alkaline phosphatase (ALP), Osteocalcin (OC), Bone Sialoprotein (BSP), and Collagen type 1.[2] Differentiation and osteoblast maturation expression markers such as collagen type I, alkaline phosphatase (ALP), bone sialoprotein (BSP), OPN, and OCN could be utilized to evaluate the osteoblastic differentiation process in experimental models.[3]

Natural substances are now being employed as an alternative to complementary therapy and alternative therapeutic ingredients that have been used around the world. Plants are employed as an alternative therapeutic product in several countries. According to the World Health Organization (WHO), 70–90 percent of pedestrians in poor nations utilize natural products to meet their health needs.[4]Alpha-Mangostin (α-mangostin) used in this study is one of the components of mangosteen peel (Garcinia mangostana) with various benefits, one of which is as an anti-inflammatory agent.[5] The mechanism of α-mangostin as an anti-inflammatory agent could be from inhibition of NO, ROS, IL-1α, and TNF-α. Besides that, the anti-inflammatory effect of α-mangostin could also inhibit NF-κβ and COX-2 selective pathways by simultaneously blocking iNOS.[6]In a study by Utari Kresnoadi and Tika Raharjo[7], the effect of α-mangostin was said to increase osteoblast production, but no study has been made about the relationship between inflammation and osteogenesis. To achieve this inflammatory response at the molecular level, LPS was administered in vitro towards osteoblast cell culture to induce an increase in pro-inflammatory cytokine expression, which in this study IL-1α was chosen.[8]Furthermore, in osteoblast cell cultures that have been induced by LPS would be treated with α-mangostin and the gene expression would be examined with Real-time PCR for ALP gene expression. The markers used in this study are IL-1α and ALP which are early markers in the inflammatory and osteoblast maturity processes, thus administration of LPS and α-mangostin within the experiment would show the gene expression.

MATERIAL AND METHODS

Ethical Clearance Certificate for this study from the Health Ethics Commission, Faculty of Dental Medicine, Universitas Airlangga (260/HRECC.FODM/V/202). This study is an in-vitro laboratory experimental study towards osteoblast cell culture, by analyzing IL-1α inflammatory marker and ALP osteogenic marker on osteoblast cell culture with and without treatment.

Osteoblast cell 7F2 is obtained from Food Industry Research and Development Institute, Taiwan (ATCC CRL-12557). The osteoblast cell was derived from the bone marrow of Mus musculus. The lipopolysaccharide used was from Escherichia coli (L2630-10MG) with Sigma Aldrich. Alpha Mangostin brand used from Sigma Aldrich (M3824) α-mangostin ≥ 98% (HPLC) products. The concentration of LPS and α-mangostin was determined from MTT assays as a preliminary study, LPS concentration result showed that at 10 ng/ml, no cell toxicity occurred. The optimal concentration of α-mangostin in the cell was 5 µg/ml. The expression of IL-1 inflammation marker and ALP osteogenesis marker caused by LPS towards 7F2 osteoblast cell was investigated using real-time PCR in this work.

The samples were divided into 4 groups: 1) Osteoblast cells culture with osteogenic medium (C) 2) Osteoblast cell culture with osteogenic medium and addition of lipopolysaccharide (E1) 3)

Osteoblast cell culture with osteogenic medium and addition of α-mangostin (E2) 4) Osteoblast cell culture with osteogenic medium and addition of lipopolysaccharide and α-mangostin (E3).

Then 7F2 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) from Sigma Aldrich (D6429) with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100µg/mL streptomycin. The osteogenic medium utilized was added with ascorbic acid 2-Phospate from TCI (A2521) and β-glycerophosphate (Gly) from Sigma Aldrich (G9891-100G).

The materials used for Real-time PCR are AriaMX Real-Time PCR Mix (G8830-64001), Serial number (MY20275218) which consist of dNTP (ATP, CTP, TTP, GTP), MgCl2, and Taq Polymerase, DW sigma (Nuclease Free water. With fold change (Delta CT) = 2^ddCt. Inflammatory marker IL-1α (107 bp) with forward primer TTGAGTCGGCAAAGAAATCAAG and reverse primer GAGAGATGGTCAATGGCAGA. Osteogenesis marker ALP (103 bp) with forward primer TTGGTGGTCACAGCAGTTG and reverse primer GACGTTCCGATCCTGAGTG.

RESULT

IL-1α Gene expression as an inflammatory marker

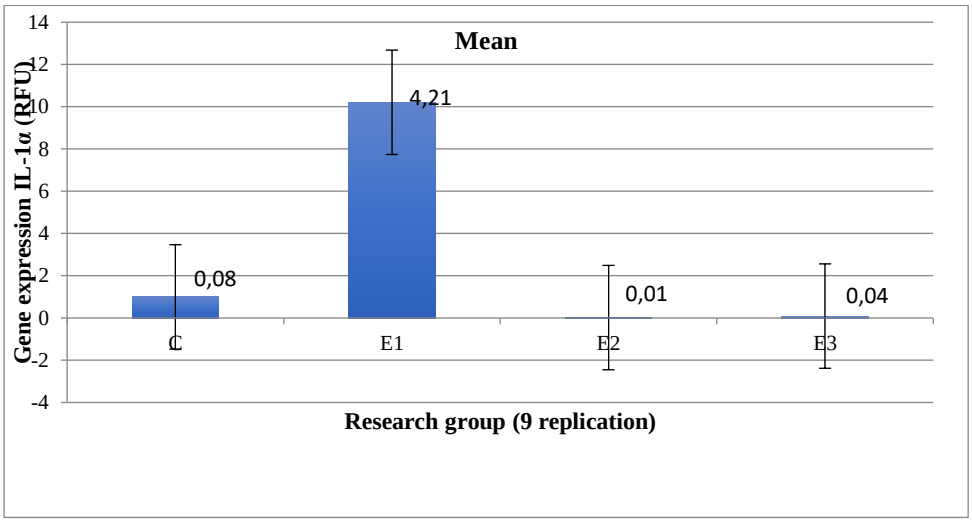

Figure 1: IL-1α expression gene.

Caption :

C : control group with osteogenic medium

E1 : treatment group with LPS

E2 : treatment group with α-mangostin

E3 : treatment group with LPS and α-mangostin

Real-time PCR running process with the K2 control group which is 7F2 cell with osteogenic medium, obtained a mean result of IL-1α gene expression in group K1 (1 ± 0,08), followed by group P1 which had treatment with LPS had the highest mean (10,21 ± 4,21), and relatively higher compared to the control group, then the mean result of group P2 which was the treatment group with α-mangostin (0,02 ± 0.01), and lastly in treatment group P3 with LPS and α-mangostin (0,09 ± 0,04). The following is the mean gene expression result of IL-1α.

ALP Gene expression as osteogenesis marker

To examine ALP gene expression with control from group K2, which is 7F2 cell with osteogenic medium, the mean result of ALP gene expression was ALP in group K2 (1 ± 0,35) was somehow similar compared to group P3. In group P1 which was treated with LPS obtained a mean result of (0,93 ± 0,26), then the mean result of treatment group P2, with α-mangostin was (0,6 ± 0,47), and lastly, the mean result in treatment group P3 with LPS dan α-mangostin was (1 ± 0,57). The following is the mean result of ALP gene expression.

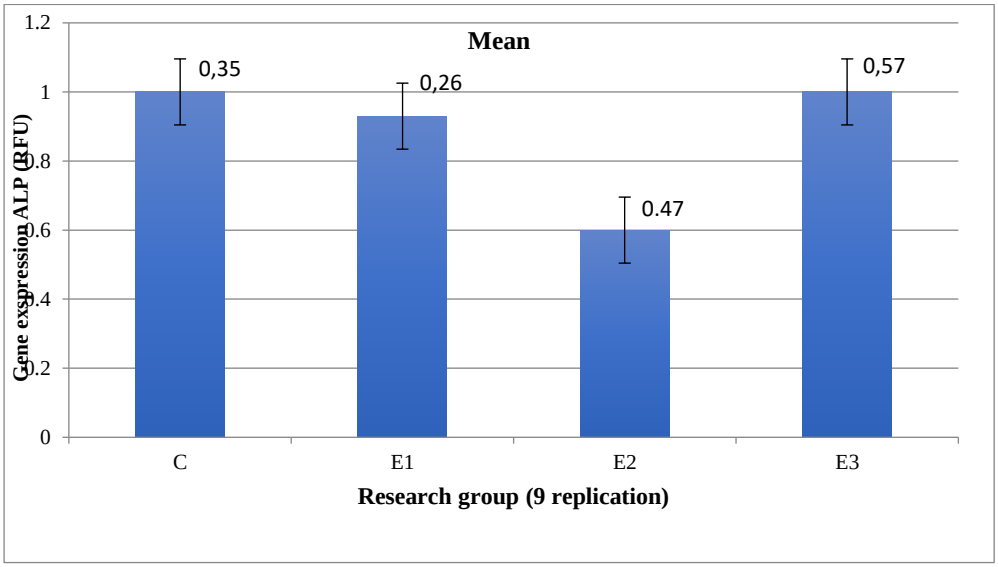

Figure 2: ALP expression gene.

Caption :

C : control group with osteogenic medium

E1 : treatment group with LPS

E2 : treatment group with α-mangostin

E3 : treatment group with LPS and α-mangostin

Statistical Analysis towards IL-1α and ALP gene expression

Based on the result of IL-1α expression gene, a normality test was conducted using the Shapiro-Wilk test. The homogeneity test was conducted with the Levene test, which showed that the data variants were not homogenous. Then Welch test and the Tukey HSD test, showed that there was a significant difference in the study group that was given LPS compared to the other treatment groups.

Table 1: Statistical analysis of IL-1α gene expression.

Group	Mean	SD	Saphiro Wilk test	Levene test	Welch test
Control with osteogenic medium (K2)	0,75	0,08	0,583	0,001**	0,001*
LPS (P1)	10,2	4,21	0,056		
α-mangostin (P2)	0,02	0,01	0,537		
LPS + α-mangostin (P3)	0,09	0,04	1,000		

*p-value<0.05in different test

**p-value>0.05on normality test / homogeneity test

Table 2: Tukey HSD test of IL-1α gene expression.

Test group	Research group	Tukey HSD test
Control with osteogenic medium (K2)	LPS (P1)	**0,009**
	α-mangostin (P2)	0,984
	LPS + α-mangostin (P3)	0,988
LPS (P1)	Control with osteogenic medium (K2)	**0,009**
	α-mangostin (P2)	**0,006**
	LPS + α-mangostin (P3)	**0,006**
α-mangostin (P2)	Control with osteogenic medium (K2)	0,984
	LPS (P1)	**0,006**
	LPS + α-mangostin (P3)	1
LPS + α-mangostin (P3)	Control with osteogenic medium (K2)	0,988
	LPS (P1)	**0,006**
	α-mangostin (P3)	1

*p-value<0.05in different test

In the ALP gene expression, a normality test was conducted with the Shapiro-Wilk test, where the data was distributed normally. Followed by homogeneity test with Levene test, showed homogenous data variants. According to the ANOVA test result, meaning there is no significant difference in ALP gene expression in the study group.

Table 3: Statistical analysis of ALP gene expression

Group	Mean	SD	Saphiro Wilk test	Levene test	Anova test
Control with osteogenic medium (K2)	1	0,35	0,092	0,306**	0,725*
LPS (P1)	0,93	0,26	0,595		
α-mangostin (P2)	0,60	0,47	0,780		
LPS + α-mangostin (P3)	1	0,57	0,141		

*p-value<0.05in different test

**p-value>0.05on normality test / homogeneity test

DISCUSSION

Osteoblast 7F2 cells are the cells that are widely used in bone research, because they can express several osteoblastic genes (RUNX2, Osteocalcin, PTH1R), alkaline phosphatase activity[9],and the ability to deposit mineral matrix in vitro or in vivo.[10][11]Several studies with these cells have been carried out to select different levels of osteogenic potential, and have been widely used as model systems in bone healing.[12][13]

The osteoblast maturation process also begins with the inflammatory process first to trigger active osteogenesis cells. In this study, 7F2 osteoblast cells were induced by using LPS to trigger inflammation in the cells.[14] The inflammatory mediator used in this study is IL-1α, because in the early stages of inflammation.[15]In this study, the osteogenesis marker ALP was used, because the role of ALP in mineralization is seen in the expression of gene phases during osteoblastic differentiation and calcified cartilage growth plate in the early stages of osteogenesis.[16]As development progresses, when other genes (eg: osteocalcin) are regulated, ALP expression decreases.[17]

Alpha Mangostin used in this study is one of the ingredients of the mangosteen peel (Garcinia mangostana) which can be used as an anti-inflammatory and to accelerate the process of osteogenesis.[18] In vitro α-mangostin was shown to be able to reduce LPS induction on the synthesis of pro-inflammatory cytokines IL-1 and TNF- α.[19]In general, IL-1α, IL-6, and TNF-α act as systemic pro-inflammatory markers. The production of IL-1α, IL-6, and/or TNF-α has shown a positive correlation with the occurrence of bone resorption or bone loss by stimulating the NO synthesis pathway (iNOS).[20]Based on the results of the study, it was found that the highest IL-1α gene expression was found in the treatment group with LPS induction (P1), the IL-1α gene expression was found to decrease with the administration of α-mangostin in both P2 and P3 groups. This is because LPS can induce inflammation in osteoblast cells, thereby triggering the emergence of pro-inflammatory cytokines, one of which is IL-1α.[20]

Then the expression of the IL-1α gene was found to decrease with the administration of α-mangostin as in the group that received treatment with α-mangostin (P2) and the group that received treatment with LPS and α-mangostin (P3), this is following research conducted by Liu et al., 2012 that in vitro α-mangostin can reduce LPS induction of pro cytokine synthesis. IL-1α inflammation. α-mangostin which can inhibit intracellular ROS activity, inhibition of intracellular ROS activity, then decreased secretion of IL-1α and TNF-α will reduce COX-2 expression which is the cause of inflammation.[19] Osteogenic differentiation can be demonstrated by measuring alkaline phosphatase (ALP) as an early marker and osteocalcin and osteopontin as a late marker.[21] The signaling pathway involved in bone formation is the Wnt/β-catenin pathway. Stimulation of this pathway will induce RUNX-2 as a major regulator of osteogenesis. RUNX-2 regulates the expression levels of osteogenic marker genes, such as ALP, OP, type I collagen, BSP, and osteocalcin.[22]Furthermore, the process of osteogenesis in osteoblast cell culture will be marked by the emergence of these markers.[12][13]

According to the results, it was found that the ALP gene expression in the treatment group that was closest to the value of the control group was the group that received treatment with LPS and α-mangostin (P3), while the group that received treatment with α-mangostin alone (P2) found a decrease in ALP expression. It was found that the ALP gene expression decreased in the treatment group that received only α-mangostin. The reason could be due to several factors, first, the IL-1α gene produced by these osteoblasts will decrease in the first 24 hours to induce osteoblast maturation, but in the P2 group, α-mangostin alone without prior LPS induction, so that the inflammatory product (IL-1α) produced is then inhibited too early by giving α-mangostin through ROS inhibition, whereas inflammation (IL-1α) is also needed by cells to trigger an inflammatory response and initiate inflammation process of osteogenesis.[23][24]It could also be due to the ineffective concentration of α-mangostin used in this study because there has been no research on α-mangostin to increase the osteoblast maturation process in osteoblast cells, so it is hoped that in future studies

higher and more diverse concentrations can be used for ALP gene expression. as a marker of osteogenesis can be better.

The 7F2 cells used in this study are osteoblast cells that have undergone mineralization so that ALP should be expressed in the early process of osteoblast maturation, and along with its development process, ALP gene expression will decrease[16], ALP gene expression after treatment does not significant differences were found between the research groups. Another factor in this study, α-mangostin may not have been able to provide a maximum effect on ALP gene expression because in this study the time of administration of α-mangostin for 24 hours still did not affect the osteoblast cells. In the study by [9], was said that 7F2 osteoblast cells began to differentiate on day 4 and ALP gene expression began to appear in the early phase of cell differentiation. This is also supported by the research of Widyowati[25] which said that the expression of the ALP gene in stimulated 7F2 osteoblast cells began to appear on day 4.

CONCLUSSION

There are decreases in IL-1α and ALP gene expression after α-mangostin administration. From this study, it can be concluded that α-mangostin can reduce inflammation which is characterized by decreased expression of the IL-1 gene but has not shown any effect on osteogenesis.

CONFLICT OF INTEREST

The authors declare there is no conflict of Interest.

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