The Potential of α-mangostin on TNF- α and OSX Expression Post Inflammation Induction on Osteoblast: An Experimental In Vitro Study

Amalia Fauqiah Azhari[1], David Buntoro Kamadjaja[2], Andra Rizqiawan[2]*, Pratiwi Soesilowati[3], Retno Widyowati[4], Kei Tobiume[5]

1. Resident of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Indonesia
2. Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine, Universitas Airlangga, Indonesia
3. Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga, Indonesia
4. Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia.
5. Department of Oral and Maxillofacial Surgery, Graduate School and Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.

Abstract

Tooth extraction may trigger an inflammatory response of alveolar bone resorption. Therefore, socket preservation is needed after tooth extraction by placing biomaterial that could maintain original alveolar bone height and width until implant placement. The mangosteen pericarp is one of natural substance that many contains of alpha- mangostin which have antioxidant, antibacterial, and anti-inflammatory. Many research was carried out for speed up healing process by decrease inflammation and increased bone healing following tooth extraction, one of them it used alpha-mangostin.

The aims of this study are to determine the direct potential of alpha-mangostin to affect the expressions of TNF-alpha as inflammatory marker gene and Osterix (OSX) as ostegenesis marker gene on osteoblastic 7F2 cell culture.

Lipopolysaccharide-treated 7F2 osteoblast cell culture with or without alpha-mangostin are subjected to quantitative RT-PCR for TNF-alpha and Osterix. This study uses a post-test-only control group design by comparing the differences of marker expressions. Statistical analysis is determined with one-way ANOVA test.

The alpha-mangostin promoted both TNF-alpha and Osterix expressions post lipopolysaccharide induction on osteoblaast 7F2 cell culture. The highest TNF-alpha gene expression was found in group that induced lipopolysaccharide and alpha-mangostin, and had significant difference in TNF-alpha expression between the group. The highest Osterix gene expression was found in group that induced lipopolysaccharide and alpha-mangostin, but there was not a significant difference in Osterix expression between the group. There is no effect of alpha-mangostin to suppress inflammatory cytokine production but promoted a process of osteogenesis on inflammatory osteoblast cells.

Experimental article (J Int Dent Med Res 2022; 15(4): 1574-1579)

Keywords: α-mangostin, TNF-α, Osterix, Osteoblast cells, lipopolysaccharide, Human, Health.

Received date: 21 August 2022 **Accept date:** 10 September 2022

Introduction

Tooth extraction triggers a traumatic inflammatory response and alveolar bone resorption in a vertical and horizontal dimension, making it harder for following prostheses such as a dental implant. Therefore, extraction sockets are necessary to maintain the volume of tooth socket dimension can be preserved until implants applied that known as socket preservation. The most widely used graft material such as graft block, membrane, or Concentrated Growth Factor (CGF) for purpose of socket preservation can decrease excessive inflammation and accelerate bone regeneration.[1] Therefore, a lot of research has been done using alternative materials besides graft for socket preservation.

One of them is mangosteen pericarp (*Garcinia mangostana* L.), of which content xanthone shows had anti-inflammatory and antimicrobial features.[2] A xanthone derivate α-

Corresponding author:
Andra Rizqiawan
Faculty of Dental Medicine, Universitas Airlangga
St. Mayjen. Prof. Dr. Moestopo No. 47, Surabaya, Indonesia.
E-mail: andra-r@fkg.unair.ac.id

mangostin can be developed during the bone healing process of socket expected may reduce inflammation, enhancing osteogenesis through osteoprogenitor modulation.[3,4]

The inflammation phase reached the peak 24 hours after injury, and then pro-inflammation cytokines activated such as interleukin-1 (IL-1), IL-6, IL-11, IL-18, and Tumor Necrosis Factor-α (TNF-α).[5] The osteoprogenitor cell will induce some osteogenesis-related molecules which may be the target of pre-natural materials. Such materials are now being employed as an alternative therapeutic ingredient and complementary therapy that has been used around the world.[6] *Garcinia mangostana* L. *(mangosteen*) is a widely tropical fruit tree in Southeast Asia such as Thailand, Malaysia, and Indonesia.[7] Alpha-mangostin is one of the components of mangosteen pericarp with various benefits, one of which is an anti-inflammatory agent.[8] There are several commercial products of α-mangostin being marketed by sigma-aldrich, TCI and cayman chemicals in the form of crystalline solid (1,3,6-trihydroxy-7-methoxy-2,8-bis(3-methyl-2-butenyl)-9H-xanthen-9-one).[9]

Osteoblast is well characterized by the specific gene expression profile in early phase of osteoblast differentiation and bone formation, including Runt Related Transcription Factor-2 (RUNX2) and Osterix (OSX or called SP7).[6]

Alpha-Mangostin has anti-inflammatory activity by inhibiting the production of intracellular Reactive Oxygen Species (ROS) activity that regulate pro inflammatory mediators. And then, pro-inflammatory cytokine can promoting osteoclast formation to cause bone resorption and enhance bone formation in periodontal tissue.[9] Thus, it is almost importance to find new materials that can inhibit bone resorption and excesive inflammation response by controling chemical mediators of inflammation.

This study aimed to evaluate α-mangostin effect on TNF-α expression that represents inflammation process and OSX expression that represent osteogenesis marker on osteoblast cell culture medium. It's hoped the research may prove occurrence of the process of accelerating bone osteogenesis on the inflammatory process after tooth extraction.

Materials and methods

Sampling criteria

In this study is a post-test only control group design using 7F2 osteoblastic cell line (CRL-12557 from bone marrow of *Mus musculus* C57BL/6 calvaria were purchased from ATCC and maintained in Dulbecco's Modified Eagle's Medium (Sigma Aldrich, Inc., St. Lois, USA, Lot-RNNJ7898), 10% fetal bovine serum (Invitrogen Corporation, Massachusetts, USA, Lot 2260082), and 100 units/mL penicillin-streptomycin (Sigma Aldrich, Inc., St. Lois, USA, Lot -049M4856V). The osteogenic medium was further supplied with ascorbic acid 2-phophate (Sigma Aldrich Inc., St. Lois, USA, RN113170551) and β-glycerophosphate (Sigma Aldrich Inc., St. Lois, USA, Lot SLCD0875). The polysaccharide (LPS) used was from *Escherchia coli* (Sigma Aldrich Inc., St. Lois, USA, M3824). α-Mangostin used in concentration 98% HPLC (Sigma Aldrich Inc., St. Lois, USA, SLBZ4723). The materials primary sequence for marker genes OSX and TNF-α which of used for Real-Time PCR are SuperScript III One-Step Real-Time PCR with Platinum (Invitrogen,Van Alley,USA,Lot1716560), See Table 1.

Experimental Analysis

Experimental in vitro analyzed using 7F2 cells were taken 1ml of the stock, and then do thawing process in the osteogenic culture medium at placed on 10-cm petri plate in 10mL of DMEM + 10% fetal bovine serum + 1% penicillin-streptomycin + 250c L-ascorbid acid 2-phosphate (AA2P) + 10 μM β-glycerophosphate (Gly) and then incubated at temperature of 37^0C in 5% CO for 72 H. The growing 7F2 cells in 80% confluent monolayer was formed and divided into four group sample: 1) osteogenic medium (P2), 2) osteogenic medium with LPS (P3), 3) osteogenic medium with alpha-mangostin (P4), 4) osteogenic medium with alpha-mangostin and LPS (P5).

For the treatment group 7F2 cell culture was treated with LPS (10 μg/ml) for 24 H and given 5 μg/ml Alpha-mangostin, that incubated for 24hours in an incubator at 37^0C. After 24 hours, each sample was extracted and the RNA using the Total RNA Purification Kit (Norgen, 17200, Canada) and then converted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad, Laboratories,Inc,California,USA) consist of 0,2 nM/unit dNTPs, 0,5μM/unit per primary pair and

0,5 Dream Taq-Hot-start DNA polymerase (Thermofisher scientific,Massachusetts, USA, Lot007876) using T100 Thermal cycle (Bio-rad, California, USA). Real-time PCR using SsoFast EvaGreen Supermix (Lot172-5200, Bio-Rad Laboratories Inc, USA) was showed on 7F2 osteoblast cell culture to get of inflammatory TNF-Alpha and osteogenesis markers of Osterix. Each sample had replicated 3 times. The reactions were performed as follows.

Real-Time PCR

For analyzed Real-time PCR in this research using CFX96 Deep well machine (Bio-Rad, Laboratories, Inc, California, USA) was performed with the following condition 95^0C for 5 minutes (40 cycles), 95^0C for 30 seconds, 63^0C for 20 seconds, and 72^0C for 40 seconds. The primer sequence was produced by Macrogen Singapore. (Table 1)

Marker gene	Primary sequence		
Osterix	F R	*GCCTACTTACCCGTCTGACTTT* *GCCCACTATTGCCAACTGC*	131 bp
TNF- Alpha	F R	*GGGGCCACCACGCTCTTCTGTC* *TGGGCTACGGGCTTGTCACTCG*	155 bp
Rpl13a	F R	*GCTTACCTGGGGCGTCTG* *ACATTCTTTTCTGCCTGTTTCC*	149 bp

Table 1. Table marker gene and primary sequence.

Statistical methods

This research was analyzed using SPSS (*Statistical Package for the Social Science Software*)17.0 edition, Version 26, IBM Corp., NY, USA). Each study group was in descriptive data, then the normality test used Saphiro-wilk test (P-value of>0,05=normal distribution). Homogeneity test used Levene test (P-value of>0,05= homogenous data). After that, a comparative-statistic test was done between each study group using one-way ANOVA (P-value of > 0,05 were considered not significant difference.

Results

This study used 4 groups of 7F2 osteoblast cell culture as sample that consist of 1 control groups medium control group with osteogenic (C) and 3 treatments groups (osteogenic medium group with LPS/P1, osteogenic medium group with α-mangostin /P2 and osteogenic medium group with LPS and α-mangosteen /P3) then incubated at a temperature of 37^0C in 5% CO2 for 24 hours. The concentration of LPS and α-mangostin was determined from MTT assays as a preliminary study. The result showed that LPS at 10 µg/ml, no cell toxicity occurred. The optimal concentration of α-mangostin for use in the cell is 5 µg/ml.

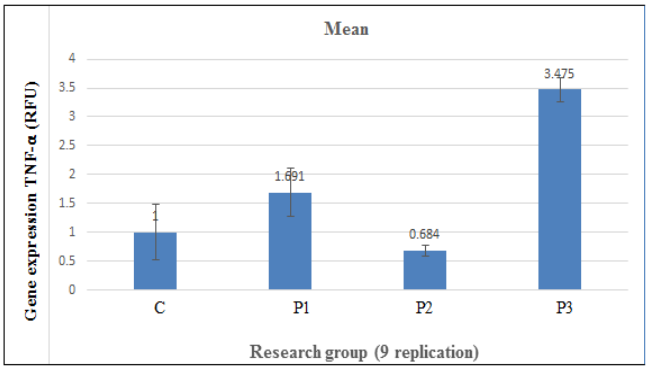

Figure 1. Bar chart of TNF- α expression gene.
Caption:
C: Control group with osteogenic medium
P1: Treatment group with LPS.
P2: Treatment group with α-mangostin.
P3: Treatment group with LPS and α-mangostin.

Figure 1 shows the results of TNF-Alpha expression on group C, P1, P2, and P3. The result of Real-Time PCR TNF-Alpha with control group osteogenic medium as a control had TNF-Alpha gene expression increased in all group that treated with Lipopolysaccharide (LPS). The highest expression of TNF-Alpha gene was found in group P4 (7F2 Osteoblast cell culture with osteogenic medium added with Lipopolysaccharide and Alpha-mangostin).

Group	Mean	SD	Saphiro Wilk test	Lavene test	Anova test
Control with osteogenic medium (C)	1.00	0.47	0.100*	0.054*	0.001*
LPS (P1)	1.69	0.42	0.071*		
α-mangostin (P2)	0.68	0.09	0.804*		
LPS+α-mangostin (P3)	3.47	0.21	1.00		

Table 2. Statistical analysis of TNF-α gene expression.
*p-value < 0.05 on lavene test = in different test
**p-value > 0.05 on normality test/homogeneity test.

Based on the one-way ANOVA result (Table 2), the treatment in all group had significant differences (*P*<0,05).

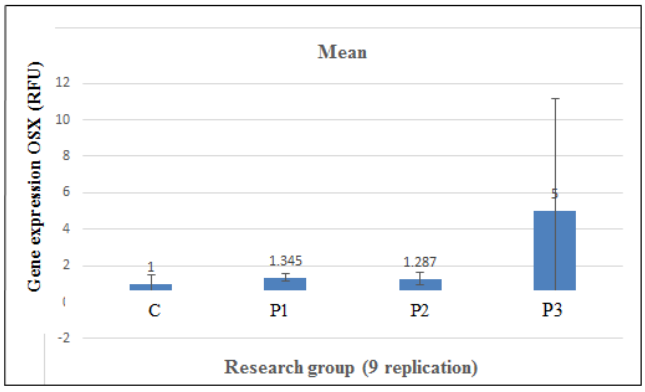

Figure 2. Bar chart of Osterix (OSX) expression gene.
Caption:
C: Control group with osteogenic medium.
P1: Treatment group with LPS.
P2: Treatment group with α-mangostin.
P3: Treatment group with LPS and α-mangostin.

Figure 2 shows the result of OSX expression on group C, P1, P2 and P3. The results of Real-Time PCR OSX with control group osteogenic medium as a control, OSX gene expression increased in P1 and P3 groups that treated with lipopolysaccharide and decreased in group P2 (7F2 osteoblast cell culture at medium osteogenic added with Alpha-mangostin). According from analyzed, the highest expression of OSX gene was found in group P3 (7F2 osteoblast cell culture with osteogenic medium added with lipopolysaccharide and alpha-mangostin).

Group	Mean	SD	Saphiro Wilk test	Lavene test	Anova test
Control with osteogenic medium(C)	1	0.47	0.100*	0.003*	0.491**
LPS (P1)	1.34	0.20	0.187*		
α -mangostin (P2)	1.28	0.36	0.500*		
LPS+α - mangostin (P3)	5.02	6.10	0.270*		

Table 3. Statistical analysis of OSX gene expression.
*p-value<0,05 in different test
**p-value>0,05 on normality test/ homogenity test.

From the result of one-way ANOVA (Table 3), the treatment in all groups had no significant difference (P>0,05).

The correlation analysis of TNF-α with OSX group using Pearson Correlation test and has coefficient correlation of +0,613 as positive correlation between two variables and has a strong correlation with p-value = 0.045 (<0.05). It can be concluded that TNF-α is increasing, then OSX also increased as positive correlation. (* p-value < 0.05).

Discussion

The current study focused on the effect of α-mangostin on osteoblast cell induced by LPS to know expression of early inflammatory gene marker and different stage maturation from osteogenic marker gene expression. The 7F2 osteoblastic cell line as in vitro study models being downstream of the MSC stage that widely used in bone research to analyze ostegenesis process, because ite expresses several osteoblastic gene (RUNX2, OSX, OCN).[1,5]

In this study, 7F2 osteoblast cell line was analyzed to know effectivity of α-mangostin on increasing osteoblastic differentiation through resistance to proinflammatory cytokine secretion on osteoblast cell culture after induction with lipopolysaccharides, using inflammation marker (TNF-α) and osteogenesis marker (OSX). This study uses α-mangostin from mangosteen fruit skin (*Garcinia mangostana*) that has the potency to accelerate osteogenesis process.[7] Alpha-mangostin could hinder activity of intracellular *Reactive Oxygen Species* (ROS) which suppressed enzyme activity that activate *nuclear factor kappa B* (NF-κB) in nucleus and regulate pro-inflammatory mediator which are TNF-α, IL-1, and IL-6. Other study showed that α-mangostin can reduce induction of LPS to proinflammatory cytokine synthesis.[2,10] The following research conducted by Liu et al., 2012 that in vitro α-mangostin can reduce LPS induction of pro cytokine synthesis which can inhibit intracellular ROS activity, then decreased secretion of IL-1α and TNF-α will reduce COX-2 expression that cause of inflammation[11].

The result of this study found that the expression of TNF-α gene was increased in the treatment group with LPS induction (P3) compare with control group (P2). The inflammation on osteoblast cell induces osteogenesis maturation, that was an inflammatory environment triggered by LPS in vitro and can activate via a TLR4/Myd88-dependent manner and transcription of several proinflammatory genes such as TNF-α, IL-1β, IL-6, and nitric oxide.[11] Another study was found that LPS-stimulated inflammatory environment by cytokines secreted TNF-α and IL-1β create a cytokines- NFκB loop to potently stimulate activated of NFκB as

multifunctional transcription factor that is associated with inflammatory response and bone metabolism.[12] Further studies, have found that inflammatory stimulus causes mitochondrial damage and then consequently induced ROS release or apoptosis which is interact with NFκB signaling pathway to activate the NLRP3 inflammasome to trigger the secretion of TNF-α, IL-1β and IL-18.[13]

In this study, the significantly highest TNF-α gene expression was found in the treatment group with LPS and α-mangostin induction (P5). It caused LPS can induce inflammatory markers such as TNF-α activates through specific binding RANK-RANKL to inhibit osteoblast cell differentiation. The 7F2 osteoblastic cell line that induced by LPS and then subsequently administered α-mangostin induces excessive inflammatory response even cell apoptosis.[8] Gutierrez O *et al*, 2013 concluded that α-mangostin on lowest dosage between 6μM to 12μM had potential to hinder activities of MAPK, NFκB and AP-1, so the expression of pro-inflammatory genes reduced as a response of anti-inflammatory effect to α-mangostin.[9,14] TNF-α is proinflammatory cytokine that increased osteoblast apoptosis progenitor, so it can suppress synthesis of type 1 collagen that can hinder differentiation of osteoblast.[15] Whereas on this study, dose of α-mangostin that used had not yet been able to give optimal effect to hinder induction of inflammation by potent LPS, and then TNF-α gen marker that showed on RT-PCR result increased significantly.

OSX expression in NFκB intercepted to control after administered LPS in 7F2 cell with real time-PCR method and it was found in the treatment group with LPS induction (P3), the inflammatory environment triggered by (LPS) in vitro would suppress BMP-2-induced osteogenic differentiation through crosstalk between TLR4/mYd88/ NF-κB and BMP/Smad signaling which may enhance bone regeneration.[12] Activation of NF-κB intercepted BMP/Smad pathway has crosstalk to regulate BMP-2-induced osteogenic differentiation via smad-dependent canonical pathway then translocate into nucleus to activate transcriptional level of osteogenic genes such as Osterix, Runx2, ALP, Collagen type 1(Coll1A1), and osteocalcin(OCN) to regulation in early phase of osteoblast differentiation.[13]

Then the expression of the OSX gene was significantly increased with administration of LPS and α-mangostin (P5) compared to the group that only received treatment with LPS (P3) and α-mangostin (P4). Lim et al. (2020) showed α-mangostin can affect the expression of OSX gene markers as early factor transcript osteogenesis on inflammatory osteoblast cell was significantly higher from an early stage at 24 until 72 hours, then osteoblast cells began to differentiate on day 4 (84hours).[8] Inflammatory osteoblast cell that inducted by LPS will release two kind of cytokine that induce osteoclast-genesis, which is M-CSF and RANKL that predominant on activated osteoprotegerin (OPG) followed by osteoblast cell which related to RANKL as protection against bone reabsorption and osteolysis. Osx play a role in promoting differentiation of mesenchymal stem cells into preosteoblasts and promoting differentiation into mature osteoblast.[16,17]

The result also showed that there was significant relationship between TNF-α and OSX caused by the TNF-α group being almost optimal. It means that LPS level administered already enough or adequate to trigger early inflammation process that was marked by increased TNF- α gen on 7F2 cell culture medium. The early inflammation process will lead to an early osteogenesis process from osteoblast cell that are seen from increased OSX gen marker.[15,18] According Hess K *et al.* (2009), TNF-α with 20 mg/dl dose could stimulate osteogenic differentiation on *human mesenchymal stem cell* by inducing NF-κB signaling pathway, subsequently induce regulation of BMP-2 that produce increased expression of RUNX-2 and OSX gen marker.[19]

Theurapeutic outcome from this study showed that α-mangostin may triggering osteogenesis which can visible especially from bone recovery via inhibit osteoclast differentiation and function, so that can hold bone high-quality.[20] Clinical set-up to help promote socket prevention that conducted by research from Utari, 2018 showed that experimental laboratory-based research using 56 Cavia cobayas as specimens with randomized factorial design which is administration of the combination of mangosteen peel extract and DFDBBX can increased the number of osteoblasts, but decreased the number of osteoclasts. Another cause that also affected the increasing of osteoblast cells in this research was the participation of a graft material

combined with mangosteen peel extract.[21] The following research conducted by Liu et al., 2012 that in vitro α-mangostin can reduce LPS induction of pro cytokine synthesis which can inhibit intracellular ROS activity, then decreased secretion of IL-1α and TNF-α will reduce COX-2 expression that cause of inflammation.[21,22]

One of the limitations of the study that it is only use mature osteoblastic cell type 7F2, so the expression of inflammation that occurs in early phase cannot be seen but expression of osteogenesis especially OSX in had been seen. This study has another limitation, including limited number of samples and observation time that make varying absorption concentration of alpha-mangostin to show the anti-inflammatory effect and osteogenic potential.

Conclusions

This study concluded the administration of α-mangostin marker on inflammatory 7F2 osteoblast cell line has not potential to reduce inflammation (TNF-α markers gene), but it had increased in osteogenesis (OSX markers gene).

Ethical policy and Institutional Review board statement

Ethical clearance had been obtained from the Ethical Commission of the Faculty Dental Medicine, Universitas Airlangga, Surabaya (No.527/HRECC.FODM/XII/2020).

Acknowledgments

We express our gratitude to Faculty of Dental Medicine, Universitas Airlangga, Indonesia, for their invaluable support for the research.

Declaration of interest

The authors have no conflicts of interest regarding this investigation.

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