

## Basic Fibroblast Growth Factor Expression after Gingival Mesenchymal Stem Cell's Metabolite Provision in Lipopolysaccharide induce inflammatory bone resorption *in vivo*

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### Abstract

Normal bone is experience bone remodeling all the time where bone resorption and bone formation are balanced. Some chronic disease, such us periodontitis could affect the bone remodelling causing bone resorption is higher than bone formation. Basic Fibroblast Growth Factors (bFGF) has role on osteogenesis which can help in increasing bone formation. Gingival Mesenchymal Stem Cells's Metabolite (GM SCM) is medical wasted products from Mesenchymal Stem Cells (MSC) which has various function. Aim of this study is to investigate the metabolites of GM SC effect on bFGF expression in inflammatory bone resorption caused by lipopolysaccharide.

The 20 experimental animals were separated into four groups: control (C): 100 g PBS day 1-7, LPS group: 100 g LPS day 1-7, LPS+GM SCs' metabolite group: 100 g LPS + 100 g GM SCs' metabolite day 1-1-7, and GM SCs' metabolite group: 100 g M-GM SCs day 1-7. Escherichia Coli LPS was employed to trigger bone resorption on the calvaria of an animal model. The dose of GM SCs metabolite administered is 100 g once day through subcutaneous injection. Furthermore, on day 8, all samples were sacrificed by cervical dislocation. To count the number of bFGF positive expressions in osteoblast in the calvaria of animal models, bFGF monoclonal antibody and Diaminobenzidine (DAB) is added, resulting in a brown precipitate developing where the antibody has attached. The statistical analysis was performed to examine the significantly different between groups ( $p < 0.05$ ). The expression of bFGF was significantly decreased in LPS induced bone resorption group (LPS group), however, after GM SCs' metabolite provision, bFGF expression was significantly elevated in GMSCs metabolite and LPS induced bone resorption (LPS+GM SCs' metabolite group) with significantly different ( $p = 0.0001$ ;  $p < 0.05$ ).

The positive expression of bFGF in osteoblast was elevated after GM SCs metabolite provision in LPS-induced calvaria bone resorption in wistar rats (*R. norvegicus*) by means of immunohistochemistry examination.

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### Introduction

Normal bone is going through remodeling for the lifetime, which contains bone formation and bone resorption that take turns in homeostasis. However, some people with chronic inflammatory disease often have bone problems in dentistry field

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such as periodontitis. The bone problems occur because of an imbalance between bone resorption by osteoclast that is higher than bone formation by osteoblast<sup>1</sup>. One of the reasons bone resorption in periodontal disease occurs is because lipopolysaccharide (LPS) from certain pathogens (such as *P. gingivalis*) induce activation of Th1, Th2, and Th17 which can activate receptor activator nuclear kappa beta ligand (RANKL) pathway and elevate production of mediator inflammatory. Activation from RANK-L receptor in preosteoclasts elevate tartrate resistant acid phosphatase (TRAP), cathepsin K, 3-integrins, and calcitonin receptors<sup>2,3</sup>.

During bone remodeling, basic Fibroblast Growth Factors (bFGF) is needed in terms of osteoblast differentiation and bone formation, other than that bFGF is able to reduce bone resorption and osteoclast formation<sup>4</sup>. In mamalia, there are 22 members on FGF family. From all the members, FGF 2, 8, 9, 10, 18, and 23 which have correlation to osteogenesis process with FGF2 in autocrine/paracrine way leads to essentially conveyed in osteoblast precursors and initiated intercellular signaling via FGFR<sup>5,6</sup>.

Gingival Mesenchymal Stem Cells's Metabolite (GMSCM) is medical wasted products from Mesenchymal Stem Cells (MSC) which have already been purified and can be used for other purposes<sup>7</sup>. GMSCM is a biocompatible product because it did not cause any systemic immune reaction which means it is not pathogenic to surrounding tissue, other than that MSCM contains an abundance of cytokines such as Epidermal Growth Factor, granulocyte-macrophage-colony-stimulating factor (GM-CSF), Interleukin-4, Interleukin-10, and transforming growth factor (TGF)- $\beta$  which are able to promote dermal fibroblast proliferation<sup>8,9</sup>.

To date, there is no study which focuses on potential effects from metabolites from gingival mesenchymal stem cells metabolite that affect bFGF expression in the LPS-induced bone which experience resorption. Thus, the aim of this study is to investigate the GMSCM effect on bFGF expression in inflammatory bone resorption caused by lipopolysaccharide.

## Materials and methods

### Study design and setting

The ethical health committee of the Faculty of Veterinary Medicine, Universitas Airlangga, accepted this study protocol for animal labs with the number 2.KE.017.02.2020. This study used a true

experimental laboratory design with an analytical post-test control group. For this study, Lameshow's are utilized to determine the minimal sample formula, hence the total sample size is 20 ( $n = 5$  samples for each group). For sample selection, a blind simple random sampling approach is utilized. Male wistar rats (*Rattus Novergicus*) weighing 250-300 grams and aged 1-2 months were used in the study. All of the experimental animals were healthy and did not have any systemic changes.

### LPS-induced animal model

The 20 experimental animals were separated into four groups: control (C): 100 g PBS day 1-7, LPS group: 100 g LPS day 1-7, LPS+GM SCs' metabolite group: 100 g LPS + 100 g GM SCs' metabolite day 1-1-7, and GMSCs' metabolite group: 100 g M-GM SCs day 1-7. Subcutaneous injection of *Escherichia Coli* LPS (Sigma Aldrich, US) was employed to trigger bone resorption on the calvaria of an animal model. The GM SCM was obtained from a patent formulation owned by *Pusat Pengembangan dan Penelitian Sel Punca Universitas Airlangga* Surabaya, East Java, Indonesia. The dose of GM SCs metabolite administered is 100 g once day through subcutaneous injection across the calvaria of animal models. Furthermore, on day 8, all samples were sacrificed by cervical dislocation.

### Immunohistochemistry analysis

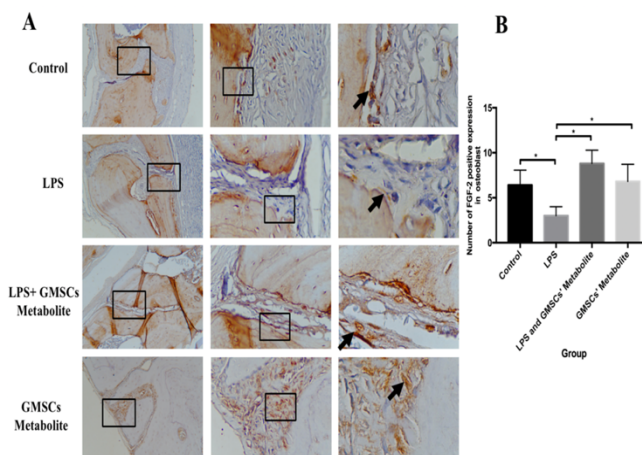
For around 10-15 minutes, the rats' calvaria were cleansed with flowing tap water. After cleaning, the calvarian is dried before being rinsed three times with 1x PBS (OneMed, Indonesia) for about 5-10 minutes each time, followed by drying. The material was then fixed in 10% neutral buffer formalin (Sigma Aldrich, US) for 4-7 days. The calvaria was then cleaned three times with 1x PBS (OneMed, Indonesia) for 5-10 minutes each time. EDTA was utilized for 14-28 days to decalcify the calvaria. After softening the calvarias, proceed to tissue processing, embedding, and sectioning with a microtome to create the Histology Pathology Anatomy (HPA) slide. To count the number of bFGF positive expressions in osteoblast in the calvaria of animal models, a 1:500 dilution of a bFGF monoclonal antibody was utilized (no.cat ab208687, Abcam, US). After that, diaminobenzidine (DAB) is added, resulting in a brown precipitate developing where the antibody has attached, which is counterstained with Hematoxylin eosin (HE) on the HPA slide. The number of bFGF positive expression in osteoblast in the calvaria was calculated using an inverted light microscope (Nikon, Tokyo, Japan) with 400x magnification in 5 fields of view viewed by two

observers. The collected data were then recapitulated and examined.

### Statistical Analysis

The data was analyzed using the Kolmogorov-Smirnov test (normality test), followed by the Levene's test (homogeneity test). If the data were normally distributed and homogenous ( $p > 0.05$ ), the differences between groups were evaluated using the One-way Analysis of Variance (ANOVA) test ( $p < 0.05$ ). The Tukey Honest Significant Difference (HSD) test was used to determine the difference between each treatment group ( $p < 0.05$ ). If the findings were normally distributed but not homogenous, the data was examined using the Kruskal-Wallis test to see if there were differences between treatments, and the Mann-Whitney test ( $p < 0.05$ ) was used to determine the difference in each treatment group. SPSS 20.0 for Mac is the latest version of the statistical package for social science (SPSS) (IBM corporation, Illinois, Chicago, US)

### Results



**Figure 1.** The positive expression of bFGF in osteoblast in the calvaria in each group (black box) were observed by means of light inverted microscope at 100x, 400x and 1000x magnification. The number of osteoblast expressed bFGF positively in each group (\* significant between groups ( $p < 0.05$ )).

The homogeneity and normality data test showed that the data homogeneous and normally distributed ( $p > 0.05$ ). bFGF expression positively expressed in osteoblast in the LPS-induced calvaria bone resorption in Wistar rats (Figure 1A). The expression of bFGF was significantly decreased in LPS induced bone resorption group (LPS group), however, after GMSCs' metabolite provision, bFGF expression was significantly elevated in GMSCs metabolite and LPS induced

bone resorption (LPS+GMSCs' metabolite group). There were significantly different between LPS induced bone resorption group (LPS group) and GMSCs metabolite and LPS induced bone resorption (LPS+GMSCs' metabolite group) ( $p = 0.0001$ ;  $p < 0.05$ ). Meanwhile, there was no significant difference in the expression of bFGF in the calvaria in PBS group (C) and GMSCs metabolite group ( $p = 0.0099$ ;  $p > 0.05$ ) (Figure 1B).

### Discussion

Bone resorption has been causing problems in various fields, thus effective and efficient therapies are being developed. Various alternative has been developed to regulate bone formation, such as the utilization of mesenchymal stem cells to regenerate bone tissue. This study was done to evaluate GMSCM provision and its effects on bone resorption in Wistar rats calvaria through observing bFGF expression in osteoblasts. Bone resorption was successfully modeled by using LPS induction to calvaria, as showed on the results that shown significantly depleted bFGF secretion post-induction. LPS generates resorption by interacting with TLR-4 thus provoking TNF- $\alpha$  secretion from cells. TNF- $\alpha$  would induce RANKL expression and further activate various pathway such as NF- $\kappa$ B in osteocytes<sup>10,11</sup>. This would lead to prostaglandins' increased activity to form osteoclasts and leading to promoted bone resorption. Preceding study elaborated the results by using RANKL primed RAW247 cells treated with LPS that shows a tremendous increase in osteoclasts number compared to the control group<sup>10</sup>. These processes are often found in various dental diseases, such as periodontitis which is one of the most prevalent oral infections<sup>12</sup>.

Inhibition of bone inflammation might be the crucial part of relieving and regenerating infected bone tissues. The formation of new bone is marked with an elevation of a plethora of growth factors exerting their functions to promote homeostasis. bFGF (FGF2) is widely known as a growth factor responsible for bone formation through osteoblast proliferation and differentiation<sup>13</sup>. bFGF also exhibit bone resorption inhibition by depressing alkaline phosphatase (ALP) activity, promoting collagen and ground substances synthesis thus leading to escalated matrix mineralization<sup>6</sup>. Bone therapy targeting bFGF secretion might be a promising alternative, as reported by preceding studies<sup>14,15</sup>.

GMSCs are stem cells that could secrete metabolites of bioactive proteins and metabolic products to the extracellular matrix<sup>16</sup>. These proteins could act as signaling, stimulation, and

inhibition to various chemical processes of cells<sup>9</sup>. This study found that metabolites significantly elevate bFGF expression in LPS-induced bone resorption. Preceding study on GMSCs metabolomics assays elaborated that metabolites comprises of various cytokines, whether pro-inflammatory or anti-inflammatory, also pro-apoptotic and pro-angiogenic factors<sup>17</sup>.

This study found enhanced bFGF expression in osteoblasts after metabolites provision in the LPS-treated group. Metabolites comprises of acetate, alanine, choline, glutamate, pyruvate, tyrosine and elses. Alanine, L-glutamate, and glucose are the prominent metabolites of stem cells. These metabolites promote cell activity, metabolism, and proliferation to enhance cell function<sup>18</sup>. These processes are also enhanced by the bioactive factors, cytokines and growth factors contained<sup>7,9</sup>. These cytokines and metabolites would ameliorate inflammatory responses occurring in the bone tissue thus leading to promoted anabolic activity in order to form new bone<sup>19</sup>. Promoted bFGF expression in osteoblast is found to be not significant in GMSCs metabolite-only treated group. The results shows that metabolites elevate bFGF expression in the safe amount to manage bone and tissue homeostasis by not excessively provoking inflammation responses in normal tissue<sup>9</sup>. Studies on bone matrix formation and mineralization by various markers are needed forth to validate and prove the bone formation capacities on GMSCs metabolites.

### Conclusions

The positive expression of bFGF in osteoblast was elevated after GMSCs metabolite provision in LPS-induced calvaria bone resorption in wistar rats (*R. norvegicus*) by means of immunohistochemistry examination.

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### Declaration of Interest

The authors declare no conflict of interest.

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