

Demineralized Freeze-dried Bovine Bone Xenograft Granules as Alveolar Bone Substitutes: A Profile Study

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Abstract

Implant placement in defective alveolar bone requires bone grafting and autogenous bone graft remains the gold standard bone material, however, it causes donor site morbidities. We explore the profile of newly developed demineralized freeze-dried bovine bone xenograft (DFDBBX). Objective: to assess profiles of DFDBBX granules.

Enzymatic protein extraction of DFDBBX were performed and specific growth factors quantified with ELISA. For compatibility study rBM-MSCs was cultured in 2.5%, 5.0% and 10% DFDBBX medium for 72 hours followed by MTT assay. Human adipose-derived MSCs was cultured in conditioned medium of DFDBBX for 2, 7, and 14 days. Expression of RUNX2 and alkaline phosphatase (ALP) were examined by immunofluorescence assay. Data was analysed statistically with significance set at p -value < 0.05 .

PDGF, VEGF, and $FGF\alpha$ levels of DFDBBX were significantly higher, BMP2 and BMP4 levels were found comparable in both groups, whereas $TGF\beta 1$ significantly lower than FDBBX. Cell viability was comparable between DFDBBX and Deproteinized Bovine Bone Material (DBBM). Runx2 and ALP expression in DFDBBX is equivalent to osteogenic medium group and detected until 14 days.

DFDBBX granules contain major proliferative and osteogenic growth factors, not cytotoxic and has osteoinductive properties equivalent to DBBM.

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Introduction

Increasing numbers of patients have demanded dental implant treatment nowadays due to its remarkable success rate. However, implant placement in defective bone may lead to functional, structural, and aesthetic problems which require horizontal ridge augmentation.^{1,2} Guided bone regeneration technique has been widely employed in alveolar bone augmentation which involve the use of bone graft materials.³

Autologous bone graft has remained the gold standard for alveolar bone augmentation because it has all the necessary properties in bone regeneration, namely, osteoconduction, osteoinduction, and osteogenesis. However, there is concern that bone grafts are limited and may lead to complications at the donor site. On the other hand, significantly lower osteoinduction and slower healing were observed for bone allograft.⁴ In order to overcome these limitations of bone grafts, synthetic bone substitutes and biological factors were developed as alternatives. Among synthetic bone substitutes and biological factors, calcium phosphate (CaP)-based biomaterials and recombinant human bone morphogenic proteins (rBMPs), which exhibited osteoinductive factors and ability to improve fracture healing, are the most widely used.⁵

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Bone regeneration involves a series of biological processes, in which many cell types and extracellular and intracellular signals are involved. Various studies have shown the involvement of several signalling pathways during embryogenic development of bone, as well as during fracture healing. The stimulation of this pathway will partially induce osteogenesis via Runx2 as the main regulator of osteogenesis. Runx2 regulates the expression levels of osteogenic marker genes, such as ALP, OPN, collagen type I, BSP, and OCN. Runx2 also regulates the expression of Osterix (Osx), which is a key transcription factor regulating the final stages of bone formation; osteoblast differentiation.^{6,7}

Recent studies on bovine bone-based materials have shown that deproteinized bovine bone mineral (DBBM) has good bone regeneration potential. It is, however, associated with poor biodegradation which may risk osseointegration of dental implant with alveolar bone.^{8,9} Demineralized Freeze-dried Bovine Bone Xenograft (DFDBBX) is, therefore, developed as an alternative bovine bone substitutes. It undergoes a demineralization process by immersion in hydrochloric acid so that it will reveal bone matrix components related to collagen fibrils called BMPs.¹⁰ DFDBBX material has osteoconductive factors when compared to bone coagulum, bone mixture, or freeze dried bone. By demineralization of bone in 0.6M hydrochloric acid prior to clot drying, the osteoinductive potential is increased as bone morphogenic protein (BMP), its osteoinductors component, becomes exposed. There are limited studies, however, on the osteogenic potential of the DFDBBX materials.

This study aim to evaluate the levels of growth factors, biocompatibility, and osteoinduction potential of DFDBBX compared to DBBM which is considered gold standard of bovine bone substitute materials.

Materials and methods

DFDBBX Growth Factors Quantification

DFDBBX granule is processed by Tissue Bank at Dr. Soetomo General Hospital, Surabaya, Indonesia. Protein content of DFDBBX were checked with ELISA assay and compared with Freeze-dried Bovine Bone Xenograft (FDBBX)

protein content. In brief, the protein content of each group was extracted using the guanidine hydrochloride method as previously described.¹¹ The levels of PDGF, VEGF, FGF α , TGF β 1, BMP2, and BMP4 in the supernatant were measured using the ELISA Kit (BT-Laboratory®) according to the manufacturer's protocol.

Cytotoxicity Assay

We cultured rBM-MSC obtained from Stem Cell Research and Development Centre, Airlangga University. This research has received an Ethical Clearance certificate from the Health Research Ethics Commission of the Faculty of Dentistry, Airlangga University, with certificate number: 386 / HRECC.FODM / VI / 2019. MTT assays were performed to assess cytotoxicity as previously described and IC50 values were calculated.¹² In brief, cells were seeded in 96 plates and cultured in alpha MEM medium with 10% FBS, Amphotericin β 1%, Penicillin streptomycin 1%, and NaHCO₃ 1-2%.) with addition of 2.5%, 5%, or 10% of DFDBBX or DBBM for 72 hours. The MTT was transformed by the living cells to a purple formazan dye which was dissolved in 100 μ L DMSO by shaking at 150 rpm for 10 min on an ELISA shaker. Finally, the relative colorimetric intensity of each well was evaluated at a 595nm wavelength.

Immunofluorescence Assay

Osteoinduction potentials of DFDBBX were assessed by comparing the number of cells expressing RUNX-2 and ALP after exposure to DFDBBX or DBBM in human Adipose derived mesenchymal stem cells (hAD-MSC) cell culture through immunofluorescence examination. Cells were obtained from the Stem Cell Research and Development Center, Airlangga University, Surabaya, Ethical Clearance certificate number: 334 / HRECC.FODM / XII / 2018. In brief, hAD-MSC were cultured at a cell density of 10,000 cells / ml in the medium with or without the addition of DFDBBX or DBBM, then observed on days 2, 7, and 14. Cells were also treated with osteogenic medium as a positive control. Expressions of RUNX2 and ALP were analyzed using FITC-conjugated anti-RUNX-2 and anti-ALP antibody (Bioss Inc.) Cells were observed with a fluorescence microscope and the number were quantified using ImageJ software.

Data analysis

Experiments were performed with replication in accordance with the minimum statistical limits required. Data are expressed as

mean ± SD and were analysed using the SPSS IBM 23. Statistical differences were evaluated using Shapiro-Wilk for normality test, the Levene's homogeneity test, and the independent T-comparison test or Mann Whitney-U test for comparison test. A $p \leq 0.05$ was considered as statistically significant.

Results DFDBBX Growth Factors Quantification

Concentrations of several growth factors related to bone growth in DFDBBX were examined with ELISA and compared with FDBBX as shown in Figure 1. PDGF, VEGF, and $FGF\alpha$ levels, which are growth factors that play a role in proliferation, are higher in DFDBBX. Significant difference was found in PDGF and VEGF, $p=0.001$ and $p=0.015$, respectively. Meanwhile, the levels of $TGF\beta1$, BMP2, and BMP4, which play a role in osteogenic induction, are less than that of FDBBX. Significant difference was found only in $TGF\beta1$, $p=0.028$.

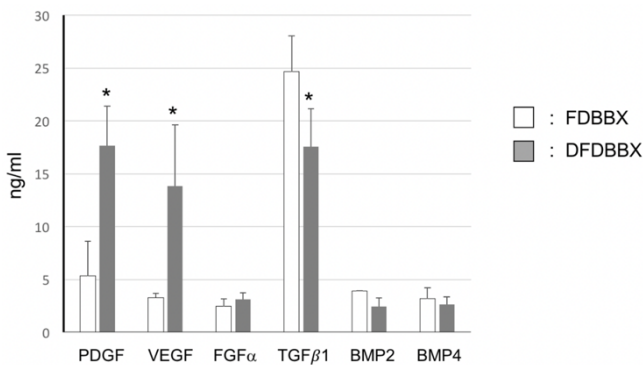


Figure 1. Growth factors quantification. Protein extracted from DFDBBX and FDBBX were measured with an ELISA specific for the indicated growth factors. Data are expressed as mean ± SD (n=5). *P < 0.05, with difference between the DFDBBX group and FDBBX group.

Cytotoxicity assay of DFDBBX

To examine the cytotoxicity effect of DFDBBX on rBM-MSCs, we exposed cells to control medium without treatment, conditioned medium of DFDBBX, and conditioned medium of DBBM with various concentrations for 72 hours and assessed cell viability using MTT assay. There were no significant differences on cell viability between control, 2.5% DFDBBX, and DBBM on all concentration as shown in Figure 2. Treatment with 5% and 10% DFDBBX reduced cell viability of cell ($p < 0.05$).

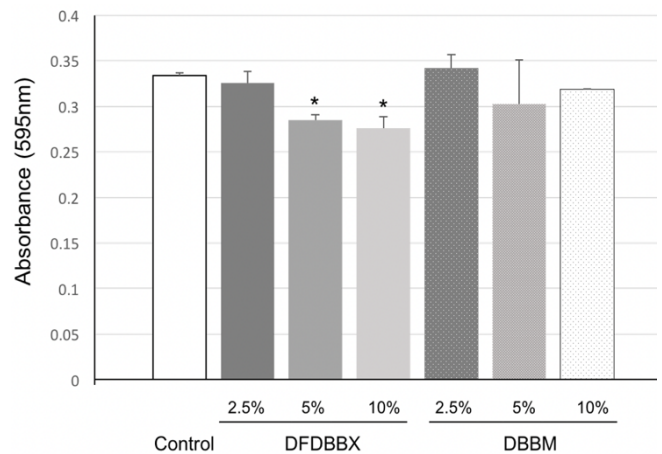


Figure 2. Effects of bone substitutes on rBM-MSCs cell viability. rBM-MSCs were cultured with DFDBBX and DBBM conditioned medium at various concentration for 72 hours. Cell cytotoxicity were assessed using MTT assay. Data are expressed as mean ± SD (n=6). *P < 0.05, with difference between the groups and untreated group.

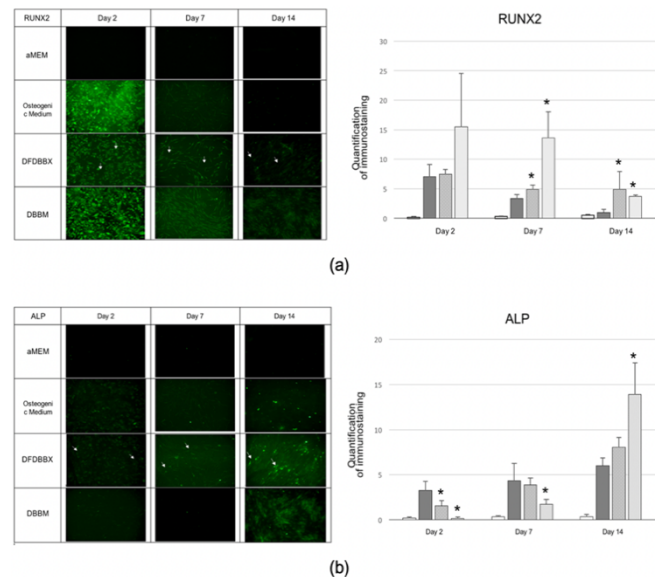


Figure 3. Osteogenic induction potential. hAD-MSCs were cultured with proliferative medium, osteogenic medium, DFDBBX conditioned medium, and DBBM conditioned medium at 2.5% concentration for the indicated time. Initial osteogenic markers were assessed using immunofluorescence. Immunofluorescence and quantification of immunofluorescence staining of (a) Runx2 at day 2, 7, and 14; (b) ALP at day 2, 7, and 14. Data are expressed as mean ± SD (n=4). *P < 0.05, compared to positive control groups.

Osteogenic induction profile of DFDBBX

hAD-MSC were cultured with the addition of osteogenic medium, DFDBBX or DBBM to examine the osteoinduction potential for the indicated time. Our results showed that all treatments increased RUNX2 at the second day. RUNX2 expression of DFDBBX treatment is sustained up to day 14 of treatment, meanwhile RUNX2 expression of osteogenic medium and DBBM is decreased on days 7 and 14. Significant differences were found in day 7 and day 14 in DFDBBX and DBBM groups compared to control ($p < 0.05$) as shown in Figure 3a. Figure 3b showed that ALP expression were increased on day 14 for all treatments and significant difference were found on the level of ALP in DBBM group.

Discussion

In this study, we explored the levels of growth factors, biocompatibility, and osteoinduction potential in DFDBBX. Previous studies of bovine bone-based xenograft materials have shown that DFDBBX has good bone regeneration potential.^{8,9} Here, we elucidated the detailed profiles of DFDBBX from its protein contents to osteoinduction potential ability. Our data could be used as a reference in further research for bone graft development, especially in the clinical setting.

First, we measured the levels of growth factor in DFDBBX and compared with FDBBX to prove that the demineralization process can release growth factors better. DFDBBX is more ready to release growth factors when implanted in the bone defects. Demineralization DFDBBX aims to remove the minerals present in the bone and leave type I collagen protein, non-collagen proteins, and growth factor, which will increase the osteoinductive properties of DFDBBX granules.¹³ Research shows that DFDBA, i.e., human bone (allograft) processed in the same way, has the ability to induce ectopic osteogenesis. This is presumably because the extracellular matrix in DFDBA contains transforming growth factor β (TGF β) in the form of Bone Morphogenic Proteins (BMPs).^{14,15}

Our results showed that PDGF, FGF α , and VEGF are found in both DFDBBX and FDBBX. This suggest that these growth factors are not derived only from osteoblast cells such as those

in autografts or allografts, but also from the remaining bone matrix in the bovine bone xenograft particles. PDGF and VEGF levels were significantly higher in DFDBBX. The loss of minerals in the demineralization process of DFDBBX causes exposure of osteonectin that binds to VEGF; thus explaining the higher levels of VEGF in DFDBBX than FDBBX.¹⁶ However, there were no significant differences in FGF α . Blaber et al., stated that guanidine HCl could promote reversible denaturation of FGF α by preventing the ongoing aggregation of proteins.¹⁷

It is noteworthy that the levels of osteogenic growth factors TGF 1, BMP2, and BMP4 were found to be lower in DFDBBX, although a significant difference was only observed in TGF 1 levels. This seems inconsistent with previous studies showing that demineralized human bone allografts have high levels of BMPs.¹⁸ We assume that this result may due to the undesired over-demineralization with acid solution performed on DFDBBX, first, during its processing and, second, during protein extraction. Previous study conducted by Pietrzak et al. stated that the demineralization process in the first 24 hours did not significantly affect the growth factor content. However, 24 hours of further acid exposure can reduce the growth factor content by as much as 50% of the optimum content.¹⁹

Cell culture in this DFDBBX biocompatibility study was obtained from rBM-MSC cultures isolated from bone marrow of a 6 weeks old Rattus Norvegicus mice from the femur that has a high level of differentiation so that their reaction patterns may be more similar to in vivo reactions compared to cell lines.²⁰ We apply DFDBBX conditioned medium with a concentration of 2.5%, 5%, and 10% referring to our preliminary study. Research by Mott et al. explains that 2% DFDBBA concentration is the lowest concentration that can be used to have a significant effect on osteoblast proliferation, concentrations below 2.5% do not have a significant effect on osteoblast cell proliferation.²¹

Our study showed that 2.5% DFDBBX is not cytotoxic to cell as stated by previous study by Vaziri et al. using DFDBBA conditioned medium in human osteoblast cell-like cultures.²² Growth factors contained in DFDBBX were also thought to play roles in increased cell proliferation. Viability were slightly decreased after 72 hours, possibly due to insufficient cell

nutrition in secondary to increased cell proliferation and differentiation of the cells; but there were no significant differences than control and DBBM, showing that DFDBBX at 2.5% is biocompatible for in vitro use.

We used human adipose derived mesenchymal stem cells (hAD-MSC) that can differentiate into various types of mesenchymal tissue to assess DFDBBX in vitro osteoinduction potential examination.²³ The osteoinduction potential of DFDBBX is compared to DBBM that have been developed commercially. DBBM is a good osteoconductor but does not have organic components and thus has weak osteoinduction potential.²⁴ The osteoinduction mechanism of DBBM is considered derived from free calcium and phosphate ions, which alter the environment and stimulate the differentiation of MSCs directly without going through the BMP pathway.²⁵ However, the disadvantage of DBBM is that it is difficult to degrade.²⁶

Runt-Related Transcription factor 2 (Runx-2) is the first transcription factor required for osteoblast differentiation that first detected in preosteoblasts and triggers the expression of major bone matrix genes during the early stages of osteoblast differentiation. RUNX-2 is best known as the main regulator of osteoblast differentiation and osteoblast marker gene expression and osteoblast function.²⁷ Our results showed that the expression of RUNX2 in DFDBBX, DBBM, and osteogenic medium groups appeared to increase at the beginning of the second day of observation, and then continued to decrease on the 7th and 14th day of observation. The level of RUNX2 induction in DFDBBX is comparable to osteogenic medium and DBBM, with a significant difference of RUNX2 expression only found in day 7 between DFDBBX and DBBM. Osteoinduction potentials of DBBM and DFDBBX are generally equivalent with observation periods. The overall regulation of osteoblast differentiation by RUNX-2 shows a shift of RUNX-2 from positive to negative regulators in osteoblastic differentiation so that the highest expression is obtained in the early stages of osteogenesis and will decrease with the osteoblast maturation process.^{28,29}

A significant increase in ALP expression ($p < 0.05$) in time-dependant manner was observed in DFDBBX and DBBM groups, with the level of expression higher than those of osteogenic medium, although it was not statistically

significant. ALP expression of DFDBBX was significantly higher than DBBM on days 2 and 7, but decreased on day 14. Collagen type I and ALP are early indicators of cellular activity and osteoblast differentiation, and ALP can be a predictor of new tissue mineralization.³⁰ Increase of ALP mRNA level is started gradually 2 days after osteogenic induction with a steady increase accompanied by an increase in osteoblast differentiation up to 14 days.

Our results showed that the initial process of osteoinduction indicated by RUNX2 expression appears to be equivalent between DFDBBX and DBBM. Meanwhile, the osteogenic differentiation process indicated by ALP expression showed the superiority of DBBM over DFDBBX. This might be due to the difference in osteoinduction mechanism between the two bone graft materials, whereby DFDBBX goes through the pathway induced by BMP-2, namely, the MAPK cascade or through Smad1/5/8 first, while DBBM in vitro stimulates differentiation of MSCs directly without going through the BMP pathway.³⁰ Birmingham et al. also stated that the osteogenic differentiation process in MSCs in vitro caused an increase in ALP levels until day 14 and would decrease after day 14 and above, which was followed by an increase in osteocalcin and osteopontin expression and resulted in calcium and phosphate deposition.³¹ Observation in this study was limited to day 14 so that the peak expressions of ALP were not observed.

Osteoinduction properties of DFDBBX examined in this study were limited to RUNX2 and ALP expression that characterize the initial process of osteogenesis. Thus, further study is needed to elucidate the osteoinduction properties in later stages by culturing the cell for a longer time to observe the expression of osteocalcin. Study on other cell types such as osteoblasts, and in vivo effects in animals to observe the direct effect of DFDBBX on osteogenesis is also needed.

Conclusions

DFDBBX granules were shown to contain important proliferative and osteogenic growth factors, biocompatible due to its biocompatibility in vitro and showed initial osteoinduction properties equivalent to DBBM. Further in vivo study is required to confirm the osteogenic potential of DFDBBX before clinical application.

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Declaration of Interest

The authors declare no conflict of interest.

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