Freeze Dried Bovine Bone (Fdbb) Scaffold Increase Expression of Alp and Col-1 in Human Umbilical Cord Mesenchymal Stem Cell (Huc-Msc) Culture

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Abstract

Repair of bone defects can be done using tissue engineering involving a combination of scaffold, stem cells, and growth factors. The natural scaffold that is commonly used is Deproteinized Bovine Bone Material (DBBM), but it is only osteoconductive and not osteoinductive and has slow biodegradation. Another alternative to the scaffold is Freeze Dried Bovine Bone (FDBB) which has better osteoconductive properties and decellularization scaffold called decellularization Freeze-Dried Bovine Bone (dc-FDBB). In the tissue engineering process, scaffold can induce the differentiation of mesenchymal stem cells and osteoprogenitor cells into osteoblasts which then form the bone matrix.

The process of osteoblast formation will affect the increase in Collagen type 1 (COL-1) and Alkaline Phosphatase (ALP) genes. The bjective to compare the expression of COL-1 and ALP on FDBB and DBBM scaffolds for bone tissue engineering. The study was an in vitro laboratory experiment on human Umbilical Cord Mesenchymal Stem Cells (hUC-MSC). There were five treatment groups, group 1 immersion of hUC-MSC on basic medium, group 2 immersion of hUC-MSC on osteogenic medium, group 3 FDBB on osteogenic medium, group 4 decellularized dc-FDBB on osteogenic medium, and group 5 DBBM on osteogenic medium. Furthermore, RT-PCR readings were carried out on the sixth and 12th days, with each sample replicated two times. Data analysis was carried out using the one-way Analysis of Variance (ANOVA) test.

As a result Group 1 showed the highest mean of ALP and COL-1 expression compared to other groups on day six and day 12. One-way ANOVA analysis test showed $p<0.05$ which indicated a significant difference between groups in the expression of ALP and COL-1. In conclusion the FDBB scaffold showed the highest ALP and COL-1 expression when compared to the DBBM and dc-FDBB groups.

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Introduction

The bone defect is a condition caused by the damage of bone cells that will affect the bone's continuity[1]. Reconstruction of mandibular defects is generally carried out by implanting bone grafts in the area of trauma. The limitation of non-vascular grafts is their susceptibility to infection; this increases the likelihood of failure as the length of the defect increases. Studies report 17% failure rates for defects of 6 cm and 75% for defects of 12 cm or more for non-vascular grafts[2]. Allografts and xenografts are bone grafts that have osteoinductive and osteoconductive characteristics, but allografts but do not have osteogenic characteristics. Autografts are the 'gold standard' in reconstructing bone, but there are some disadvantages such as limited bone donor sources, the risk of cadaver donors transmitting infectious diseases, expensive screening tests and strict regulation (the

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substances may only be processed by certified tissue banks).

Bone reconstruction using tissue engineering involves a combination of a scaffold, stem cells and growth factors[3]. The use of natural scaffolds can be in the form of xenogenic scaffolds such as deproteinized bovine bone (DBBM) with the advantage of not having the potential to be immunogenic rejection but with several weaknesses such as only being osteoconductive, not osteoinductive and the biodegradation process being very slow, thereby reducing bone regeneration[3]. Another option is the Bovine Bone Scaffold which is freeze-dried or lyophilized (FDBB). The lyophilization process can reduce the antigenic potential and is able to maintain organic components so that it is considered to still have osteoinductive properties[4]. Another technique used for the scaffold is decellularization called decellularization Freeze-Dried Bovine Bone (dc-FDBB), in which it is hoped that the cellular component known as the gel epitope, which can cause an immune and inflammatory response to the biological scaffold, can be removed.

Ideal scaffolds in bone tissue engineering are osteoinductive, which can induce the differentiation of mesenchymal stem cells and osteoprogenitor cells into osteoblasts and then continue to form the bone matrix[3]. During the process of osteoblast formation, there are several genes including collagen type 1 (COL-1), alkaline phosphatase (ALP), and osteoprotegerin (OPN) which show increased expression levels due to scaffold stimulation[5]. This study aims to analyse the osteogenic potential of the FDBB scaffold in human Umbilical Cord Mesenchymal Stem Cells (hUC-MSC) culture using osteoblastic differentiation markers COL-1 and ALP compared with DBBM and dc-FDBB.

Materials and methods

This research was conducted via laboratory experimental analysis of in vitro. The study designed a post-test only control group comparing the use of FDBB, DBBM and dc-FDBB scaffold, and analysing the expression of ALP and COL-1 markers in hUC-MSC cell cultures. This research has received an ethics-appropriate certificate issued by the Faculty of Dentistry, Airlangga University, with the ethics number 024/HRECC.FODM/1/2022.

The sample was divided into 5 groups:

- Group 1 was hUC-MSC cell culture with a basic medium
- Group 2 was hUC-MSC cell culture with an osteogenic medium
- Group 3 was hUC-MSC cell culture with scaffold freeze-dried bovine bone cancellus non-decellularization
- Group 4 was hUC-MSC cell culture with decellularization freeze dried bovine bone scaffold
- Group 5 was hUC-MSC cell culture with cancellus Deproteinized Bovine Bone Material.

In each group, observations were made at six days and 12 days. In each sample, the osteogenic markers COL-1 and ALP were be observed with two times of replication.

The research was carried out in the laboratory of the Institute Tropical Disease Centre, Airlangga University, Surabaya. Meanwhile, the production of FDBB, dc-FDBB, and DBBM was carried out at the Centre for Biomaterials and the Tissue Bank of RSUD Dr Soetomo, Surabaya.

Isolation and Culture hUC-MSC Cell

Firstly, umbilical cells were collected, then the entire cord was washed in Phosphate Buffer Saline (PBS) solution (Life Sciences, UK) three times to remove red blood cells. Then it was soaked in 70% ethanol (Sigma, UK) for 30 seconds and immediately washed again in PBS before further processing. Approximately 2-3 cm of the entire cord was removed for processing, and approximately 6 cm of the entire cord was dissected to collect arteries, veins, Wharton's jelly and cord lining. Explant cultures were collected from each region where they were weighed and cut into small pieces (~2 mm^3) with a sterile scalpel. The cells were collected and cultured on collagen-coated dishes using Minimum Essential Medium Eagle; alpha modification (α-MEM) (Gibco BRL, Gaithersburg, MD, USA), supplemented with human leukaemia inhibitory factor (10ng/mL) and foetal bovine serum (FBS) (Gibco BRL). Cell viability was calculated by trypan blue (Sigma) exclusion on a haemocytometer. The cell splitting was done using trypsin.

Preparation Scaffold

This study used three types of scaffolds which were Freeze-Dried Bovine Bone (FDBB), Deproteinized Bovine Bone Material (DBBM), and

decellularization Freeze-Dried Bovine Bone (dc-FDBB). The method used to make scaffold was the same as the previous research. FDBB, DBBM and dc-FDBB block scaffold sized 10 x 5 x 5 mm was obtained from Tissue Bank Dr Soetomo Academic Medical Hospital, Surabaya with the production date of 14 December 2021. (Figure 1, 2, 3, 4)

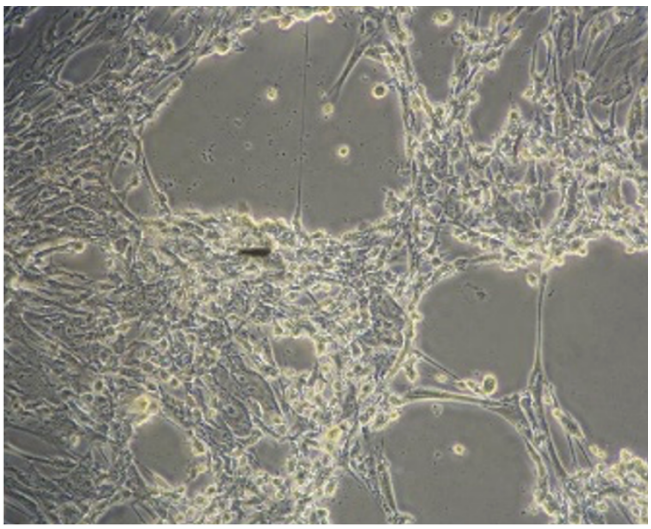

Figure 1. The microscopic of hUC-MSC cells.

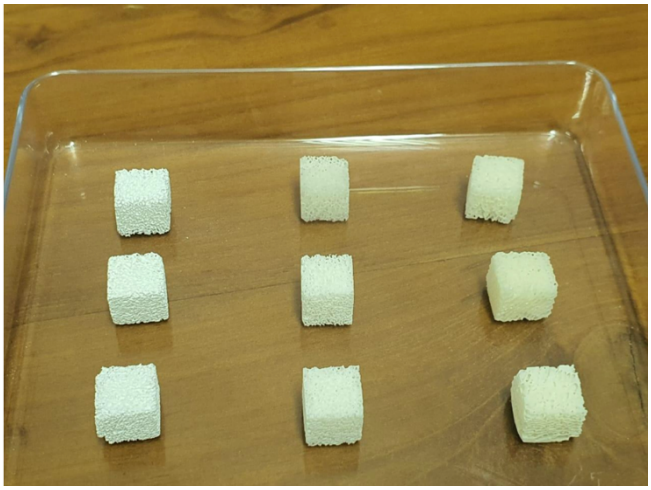

Figure 2. Scaffold DBBM, FDBB, and dc-FDBB (left to right).

Preparation of Conditiated Medium

The manufacture of conditioned medium was a modification of Asparuhova's research[5].

a. FDBB, DC-FDBB, and DBBM scaffold were rehydrated in plus growth medium (α-MEM + 2mM Glutamine, FBS, antibiotics, antifungals) with a concentration of 5 g/10mL solvent medium, for seven days.
b. After centrifugation at 600 g Force within eight minutes at temperature 20°C then obtained supernatant.
c. Supernatant was taken and filtered with a 0.22 mM filter, so that the conditioned media FDBB, DC-FDBB, and DBBM were obtained.

Primers Gene

Primers for ALP and human COL-1 were designed with the system from Primer Express TM software version 1.0 (Applied Biosystems). The probe was a short oligopeptide sequence specific to the target sequence and acted as a monitoring system for DNA amplification. Primers and probes were designed by inputting sequences to TaqMan or TaqMan MGB Assays Applied Biosystem[6](Table 1).

Gene	Primer Sequence		Primer's length
Collagen type 1	F	CGAAGACATCCCACCAATCAC	250 bp
	R	TCCCTTGGGTCCCTCGAC	
ALP	F	CCTGGACCTCGTTGACACCT	136 bp
	R	GTCCCCTGGCTCGAAGAGA	
GADPH	F	CGAAGACATCCCACCAATCAC	650 bp
	R	GAGCCCTTCCACAATGCCAAA	

Table 1. Primers and probes inputting sequences Biosystem.

RNA Extraction and cDNA Synthesis

Synthesis was carried out with iSCRIPTc cDNAc SYNTHESIS, 25R (Bio Rad Laboratories) with the following protocol:

- Mix and centrifuge the following components: extracted RNA, primer and/or random primer, and *nuclease-free water*
- Heat at 70°C for five minutes, then chill in ice water for five minute
- Carry out centrifugation for ten seconds using a *microcentrifuge.* Store on ice and add *reverse transcription*
- Mix *reverse transcription (GoScript buffer, $MgCl_2$,* PCR *Nucleotides,* RNA *sin, GoScript Reverse transcription)* to a volume of 15 µl
- Finally, mix 15 µl *reverse transcription* with 5 µl mixed RNA and primer. *Anneal* at 25°C for five minutes, then store at 4°C.

RT-PCR

Reactions were performed and monitored using the ABI Prism 7700 sequence detection system (Applied Biosystems, Rotkreuz, Switzerland, CH). ALP and COL-1 PCR reading

and optimization took place using the Real-Time PCR program GoTaq® qPCR Master Mix (Promega, USA) with mixing: qPCR mastermix, Primer Forward, Primer Reverse, cDNA template, Nuclease free water.

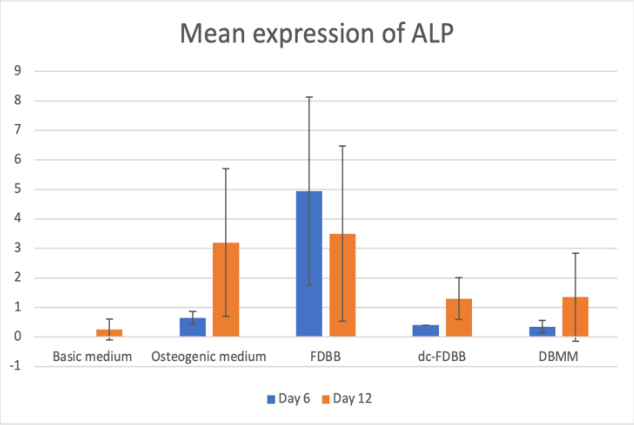

Figure 3. Mean expression of ALP in experimental and control groups on day 6 and 12.

On day 6 and day twelve the FDBB group showed a mean COL-1 expression that was relatively high compared to other groups. In the negative control group, positive control, and treatment 1, there was a significant decrease on day 12 compared to day six. Meanwhile, in the dc-FDBB and DBBM groups, there was a slight increase on day 12 compared to day six.

A one-way ANOVA test was conducted to determine differences between groups. The test showed the result $p=0.009$ ($p<0.05$) which indicated there are significant differences between groups. The analysis then continued with a post hoc test to find out the details of the differences in each group using LSD with $p<0.05$ showing that there were differences between groups.

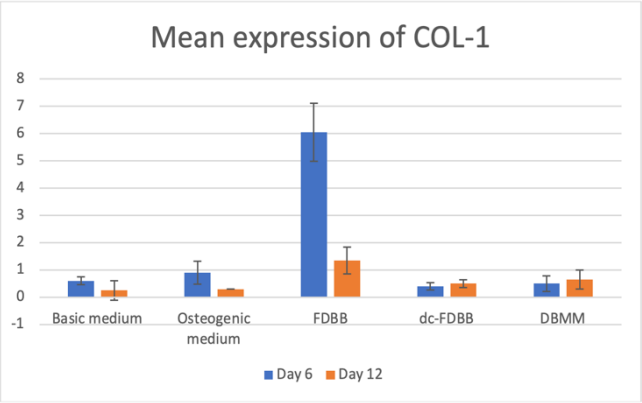

Figure 4. Mean expression of ALP in experimental and control groups on day 6 and 12.

Discussion

This research is an in vitro method using hUC-MSC media. According to Motamedian et al.[7], hUC-MSC has the characteristics of high self-repair ability and multilineage and is considered the most used source of osteoprogenitor cells. In addition, hUC-MSC has a superior proliferation rate and produces more GAG and type I collagen than Bone Marrow Stem Cell (BMSC)[6]. This study uses a scaffold conditioned medium with hUC-MSC cells with an optimal ratio of the Scaffold Conditioned Medium (CM): Osteogenic Medium (OM) ratio of 3:7 to increase osteodifferentiation optimally according to the procedure and research results from Zhong et al. which show that this ratio significantly increases the expression of the markers RUNX2, COL-1, ALP, and OCN compared to groups with other ratios[8].

The study was observed on the sixth and 12th days. According to Kamadjaja et al.[9], the increased expression of ALP and osteocalcin reflects the early processes of osteoblastic maturation, matrix deposition and mineralization. The process of osteogenic differentiation in ALP showed a maximum expression level on day 14 and was followed by an increase in the expression of osteocalcin and osteopontin leading to calcium-phosphate. Meanwhile, the expression of the COL-1 gene often occurs during the transition from the early phase to the maturation phase of osteoblasts where osteoblasts begin to form the extracellularmatrix[10].

ALP is a glycoprotein cell and assumed to be associated with ECM and mineralization during osteogenic differentiation[11]. ALP plays an important role in inducing mineralization by providing phosphate as a result of hydrolysis phyrophosphatase[12]. ALP is one of the important markers in bone regeneration used to determine the early and late phases of osteoblast cell differentiation associated with mineral deposition[13]. In the study results, ALP expression showed that group 1, which was FDBB on osteogenic media, showed the highest results on the 6th and 12th days. This is following research by Kamadjaja et al.[14] stated that FDBB had a better osteogenic level than the DBBM group. FDBB consists of organic and inorganic structures in the form of ECM which functions as an osteoconductor and osteoinductor consisting of collagen fibres and Glycosaminoglycans

(GAG), the most optimum media for cell attachment and proliferation. ALP regulation in osteoblastic differentiation is a complex signalling process. The main regulators controlling ALP in osteoblastic differentiation and chondrogenesis are the signalling of BMP/Runx2, the Osterix system, and catenin[15]. In group 1, ALP expression showed a slight decrease on day 12. This can be supported by the research of Chen et al.[13] which stated that the results of ALP expression increased gradually and peaked on the seventh day while on the 10th day it decreased. Another study by Birmingham et al.[16] stated that the process of osteogenic differentiation in MSCs in vitro show an increase in ALP levels until day 14 and decrease thereafter.

In the results of the study, the expression of COL-1 showed that group FDBB on osteogenic media had the highest results on the sixth and 12th days. The increase in COL-1 expression is an indication of its high osteoinductive potential[17]. The research of Kamadjaja et al.[14] stated that FDBB showed osteoblast differentiation and proliferation that occurred in the initial phase of treatment. This condition may be caused by the growth factor released during FDBB matrix degradation. The high expression of osteogenic markers can be caused by osteoblast differentiation in mesenchymal cells. These cells trigger the interaction of many growth factors such as BMP2, BMP4, and TGF-β which are released during matrix resorption in FDBB particles[18]. BMP activation also affects the increase in RUNX2 transcriptional activity, which induces the expression of genes associated with osteoblast differentiation and increased bone formation. In addition, TGF-β supports osteoprogenitor proliferation, early differentiation, and the association between osteoblastic lines through MAPK and the smad 2/3 selective pathway that promotes increased COL-1 expression[19].

The positive control group using osteogenic media had higher ALP and COL-1 expression compared to the negative control group. The osteogenic medium contains ascorbic acid, dexamethasone, and glycerol phosphate, which are osteoinducers highly effective in vitro. The osteogenic medium has the same osteoinduction capacity as several growth factors such as Vascular Endothelium Growth Factor (VEGF) and Bone Morphogenetic Protein (BMP). However, the expression of ALP and COL-1 in group 1, group 2 and group 3 had higher results than the positive control group. This shows that the use of scaffolds provides osteoinductive potential in tissue engineering. This condition exists because the three components of the scaffold have osteoinductive capacity. FDBB and dc-FDBB contain BMP-2 which can modulate RUNX2 expression through the signalling pathway wnt catenin and DBBM has an osteoinductive capacity because DBBM is able to stimulate cell differentiation directly without going through the BMP pathway, namely through free ions of calcium and phosphate which will activate the notch signalling pathway and result in increased osteoinductive gene expression[20].

In this study, the results of ALP and COL-1 expression of dc-FDBB showed relatively low results compared to the FDBB and DBBM groups. There are some weaknesses mentioned in previous studies, that the sterilization technology in the manufacture of decellularization is still inadequate which can cause cell growth inhibition in bone regeneration engineering[21]. In addition, it was mentioned in the study of Amirazad et al.[22] that there are challenges in the process of decellularization which allows damage to ECM, DNA, and BMP-2 proteins during washing with ionic detergents during the scaffolding process. This can affect the possibility of decreasing the potential for osteoblastogenesis through signalling pathways. Meanwhile, the DBBM group had lower ALP and COL-1 expression results than the FDBB group.[23] This can happen because, as research by Kamadjaja et al.[14] stated, DBBM has weak biodegradation capabilities and low bone regeneration capacity. The limitation of the study did not allow for the highest and lowest level of expression ALP and COL-1.

Conclusions

From the results of this study, it is concluded that FDBB scaffold has the highest osteogenic potential compared to dc-FDBB and DBBM scaffold based on the expression of osteoblastic differentiation markers COL-1 and ALP. However, some level of ALP and COL-1 show decreased numbers on day 12, and do not show the highest and lowest level of expression of ALP and COL-1. This result requires further study to optimize the analysis of results.

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Declaration of Interest

The authors declare that there is no conflict of interest in this study.

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