Freeze-Dried Bovine Bone as Xenogenic Scaffold: Does Decellularization Lower Its Antigenic Potential?

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Abstract

Deproteinized Bovine Bone Mineral (DBBM) has been widely used as tissue engineering scaffold, because of its not antigenic potential, although non-resorbable. Freeze-Dried Bovine Bone (FDBB) scaffold was developed to overcome the limitations of DBBM but it's still suspected to have antigenic potential. Therefore, to overcome the issues of antigenic potential, decellularization of FDBB was carried out (dc-FDBB). Objective: To analyze antigenic potential of FDBB scaffold with process decellularization .

FDBB and dc-FDBB scaffolds compared to DBBM scaffold as gold standard. Ten samples for each group of scaffold FDBB, dc-FDBB, and DBBM. The groups were observed for residual osteoblasts and osteocytes with HE staining using a light microscope with 40 x and 400 x magnification. DNA concentration analysis was carried out on the three types of scaffolds using a spectrophotometer. Statistical analysis was performed using IBM SPSS version 25. Data were statistically analyzed using ANOVA and Post Hoc Games-Howell tests.

There were degradation of osteocytes without nucleus and satisfactory condition of extracellular matrix geometrical structure in the FDBB scaffold. There were no osteoblasts and osteocytes with damaged extracellular matrix geometrical structures were seen in the dc-FDBB scaffold. There were no osteoblasts and osteocytes with satisfactory condition of extracellular matrix geometrical structure in the DBBM scaffold. Mean values of DNA concentration: FDBB, dc-FDBB, and DBBM were of (19.75 ng/µL ± 4.80), (16.84 ng/µL ± 6.55), and (8.72 ng/µL ± 0.65) respectively.

Comparative p value FDBB and dc-FDBB scaffolds were 0,509 (p>0,05), there were no statistically significant difference between FDBB and dc-FDBB scaffolds.

FDBB and dc-FDBB scaffolds can be used because it has low antigenic potential.

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Introduction

More than two million autografts were given to patients each year worldwide, raising concerns about the limited supply in terms of shape and quantity. Autograft provides bone cells (osteogenic cells) and growth factors that osteoinductive and osteogenic properties, without the risks of rejection and disease transmission. Disadvantages of autograft are: pain and morbidity in the donor site, limited shape and quantity, need for further surgery, hematoma, infection, longer operative time, and blood loss.[1] Many studies had been conducted to find alternatives for autograft. Allograft, xenograft, and alloplast are alternatives to overcome its limitations. An ideal bone graft substitute should be biomechanically stable, able to degrade within an appropriate time, exhibit osteoconductive, osteoinductive, and osteogenic properties and provide a favourable environtment for invading blood vessels and bone forming cells. Osteoconductive is ability of a material to structurally support bone growth. Osteoinductive is ability of a material to induce bone formation.

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Osteogenic is ability of a material to create bone intrinsically.[2]

Allograft is bone graft material derived from different individual of the same species. Allografts are well received by donor recipients with minimal rejection reactions and do not require additional surgery.[3] Unfortunately, it has a risk of disease transmission, requires a series of processes with high costs that can effect on structures and proteins that can lead to loss of osteogenic cells, and are limited in number and shape of allograft. Allograft was limited in supply since they require a donor and donor site.[4,5]

To overcome costly autograft and allograft, then developed xenograft material from other species, for example bovine bone. The first medical description of a bone grafting procedure was in 1668, when Jacob van Meekeren described a successful xenograft from a dog calvarium to a soldier's skull. In dentistry, bovine bone xenograft is often used and reveal good results. Bovine bones have a physical and chemical structures similar to human bones and are not limited in number both in terms of shape and number.[6,7] Researchers tried to produce biocompatible xenografts by carrying out various processes on bovine bone xenografts, such as freeze dried, deproteinized, and demineralized.[5]

Deproteinized Bovine Bone Mineral (DBBM) is a bone graft material that has been widely used and studied.[8,9] The bone graft material is processed through a deproteination process, which removes organic components from the bone to have properties that are more acceptable to the host body because it reduces antigenic factors.[10,11] It has a chemical composition and architectural geometry that is almost identical to that of human bone (osteoconductive) and can support new bone formation in direct contact to the graft, although non-resorbable. Freeze-Dried Bovine Bone (FDBB) is a bone graft material derived from bovine bones that have been frozen and dried or lyophilized, to maintain the physical and chemical structure and suppressing excessive antigen reactions that can occur. It has organic and inorganic components therefore it could be completely absorbed by body, it can support bone regeneration. It needed to developed an ideal graft material.[8]

Bone graft material must be free of antigenic potential. Antigenic potential is the presence of cell residues in bone graft material which are considered as antigens because they can trigger a proinflammatory response and affect the remodeling process. Nucleic acid residues can also trigger rejection reactions.[12] To produce an ideal xenograft, several processing methods continue to be developed, one of which is the process of cell cleansing or decellularization. Decellularization aims to eliminate cellular components from the original tissue including DNA and RNA, but still maintain the structure and composition of the extracellular matrix so that it is expected to reduce antigenic potential and lead to a constructive remodeling process.[13,14] Research by Bracey et al. (2018) stated that the decellularization technique can produce biocompatible and pathogen-free bone graft material.[15]

Cancellous bovine bone scaffold were use in this study because of its high vascularity and adequate porosity, with a wide area and sufficient space between the bone structure thus maximizing decellularization process. The aim of this study to analyze decellularization process could eliminate the antigenic potential of the FDBB scaffold.

Materials and methods

This research was an in vitro study comparing residual of osteoblasts and osteocytes and DNA concentration of FDBB and dc-FDBB scaffolds as research groups and DBBM scaffolds as control group. Ten samples in each group of scaffolds FDBB, dc-FDBB, and DBBM, respectively, were observed for residual osteoblasts and osteocytes with HE staining using a light microscope (Nikon Eclipse Si) with 40 x and 400 x magnification. DNA concentration analysis was carried out on the three scaffolds using a One Microvolume UV-Vis Spectrophotometer (Thermo Scientific-Nanodrop™). Production of samples of FDBB, dc-FDBB, and DBBM in Cell and Tissue Bank of RSUD Dr. Soetomo, Surabaya. Experiments, data collection, data processing and compilation of results were carried out at the Research Center of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya.

Scaffold Preparation

FDBB, dc-FDBB, and DBBM scaffolds from cancellous bovine femur bone blocks with 10x5x5 mm (Figure 1). Packing in 2 layers or packing scaffold, tightly sealed and sent for

sterilization using gamma rays.

The FDBB process: bones were soaked in a 3% hydrogen peroxide solution to remove any residual blood, fat, and bone marrow rinsed with sterile distilled water to clean the remaining peroxide solution. After washing, the beef bones were dried by freeze-drying at -80°C and dried with a lyophilizer until the moisture content was below 10%.

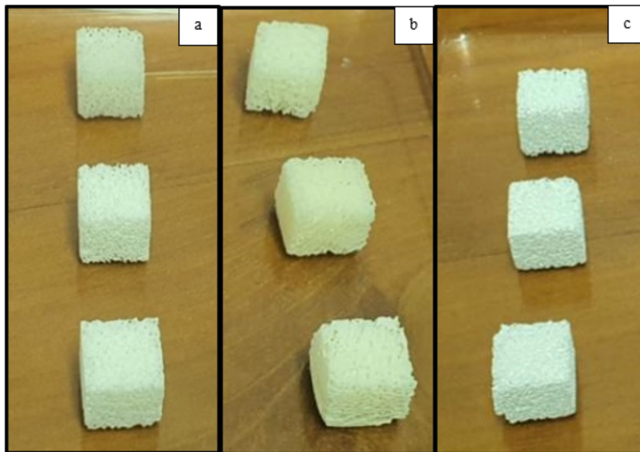

Figure 1. FDBB scaffold (a), dc-FDBB scaffold (b), and DBBM scaffold (c).

The decellularization method FDBB were using sodium dodecyl sulfate (SDS). The samples were stored at 4°C and then rinsed with phosphate saline (PBS) solution before being given SDS with a concentration of 0.5% for 1 hour. Samples were rinsed with sterile distilled water and PBS, scaffolds were placed on a stirrer and rinsed with a solution containing chloroform and ethanol, with an initial ratio of 2:1 for 24 hours.

The DBBM process: bones were washed with hydrogen peroxide and 0.9% NaCl to remove the remnants of fat. The deproteination process was carried out by burning samples at temperature of 1000°C, rinsed with sterile distilled water. The scaffold was dried in the oven at a temperature of 100°C.

Histological Evaluation of Osteoblast and Osteocytes Cells

For histological analysis of samples were decalcified with 10% EDTA, put in formalin buffer for 24 hours at room temperature. Paraffin-embedded tissue sections (4 μm) were stained with hematoxylin and eosin (H&E). Histological analysis was performed using a light microscope to see the presence of osteoblasts and osteocytes. Osteoblast cells appear with blue nucleus on the bone surface. Osteocyte cells with blue nucleus in lacunae on the extracellular matrix are pink.

DNA Quantification

DNA extraction kit uses lysis buffer B and Proteinase K to lyse cells and degrade protein. Mix well by gentle vortexing then incubate. Adding ethanol, mix by vortexing to bind to column. Apply solution WN to the column and centrifuge. Wash solution A to the column and centrifuge. Discard the flowthrough and reassemble the spin column with its collection tube. Place the column into a provided elution tube, add elution buffer B to the column. The purrified DNA sample may be stored at 4°C for a few days. To measure the DNA concentration in the research sample that had previously been extracted using a One Microvolume UV-Vis Spectrophotometer (Thermo Scientific-Nanodrop™), with a sample volume of 1-2μL.[16]

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 25. Shapiro-wilk test was conducted for data distribution normality test and Levene's test for homogeneity test. The Brown-Forsythe Anova test was carried out to see the differences among groups. The final test was Post Hoc Games-Howell.

Results

There was degradation of osteocytes without nucleus and satisfactory condition of extracellular matrix geometrical structure in the FDBB scaffold. There were no osteoblasts and osteocytes with damaged extracellular matrix geometrical structures were seen in the dc-FDBB scaffold. There were no osteoblasts and osteocytes with satisfactory condition of extracellular matrix geometrical structure in the DBBM scaffold (Figure 2).

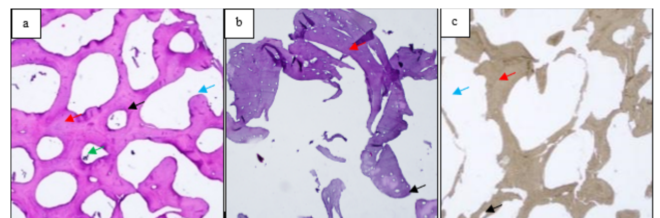

Figure 2. Microscopic imaging with light microscope with 40x magnification after HE staining (a) FDBB scaffold, (b) dc-FDBB scaffold, (c) DBBM scaffold. The green arrow indicates the

osteocyte in the lacunae, the blue arrow indicates trabeculae, the red arrow indicates extracellular matrix, and the black arrow indicates lacunae.

The highest average DNA concentration were found in the FDBB scaffold, followed by the dc-FDBB and DBBM scaffolds. The results of the quantification of the average DNA concentration in the three scaffolds can be seen in table 1 and figure 3. The average DNA purity values were 1.60, 1.63, 1.48 for FDBB, dc-FDBB, and DBBM, respectively (Table 2).

Groups	Mean of DNA Concentration (ng/µL)	Std. Deviation	Saphiro-Wilk Test	Levene Test	Brown-Forsythe ANOVA Test
FDBB	19.75	± 4.80	0.385	0.003	0.000
dc-FDBB	16.84	± 6.55	0.535		
DBBM	8.72	± 0.64	0.968		

Table 1. DNA Concentration Statistical Analysis.

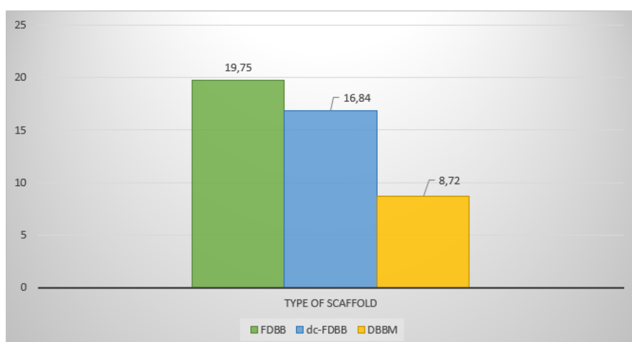

Figure 3. Mean of DNA concentration scaffolds.

Groups	N	Value
FDBB	10	1.60
dc-FDBB	10	1.63
DBBM	10	1.48

Table 2. Mean of DNA Purity Value.

Levene's test showed a significance value of p = 0.003 (p<0.05) whereas in this study the data were not homogeneous.

The results of the ANOVA test showed a significance value of p = 0.000 (p<0.05), which means that there was a significant difference between groups. Post Hoc test using the Games-Howell test to show differences between the scaffold groups. The p-value between the FDBB-DBBM scaffold groups was 0.000. The value showed a p-value<0.05. It was a significant difference between the two scaffold groups. The p-value between scaffold groups dc-FDBB-DBBM was 0.009. The value showed p-value<0.05. It was a significant difference between the two scaffold groups. Meanwhile, the p-value between the FDBB-dc-FDBB scaffold groups was 0.509. The value shows p-value>0.05. There was no significant difference between the two scaffold groups.

Discussion

The difference of decalcification process time among the three scaffold groups. It was happen due to the fact that the FDBB and dc-FDBB scaffold groups still had organic and inorganic components, therefore decalcification solution worked quickly. In the DBBM scaffold group, the duration of the decalcification process was two times slower than the FDBB and dc-FDBB scaffold groups. This was because the DBBM scaffold only has inorganic components (Hydroxypatite). It makes the tissue structure harder than other groups.[17]

The results of the examination examined by a microscope with HE scaffold FDBB staining, revealed that there was a damaged osteocyte cell residues, but on the dc-FDBB scaffold, there were no osteoblasts and osteocytes. The similiar thing also appears on the DBBM scaffold, there were no osteoblasts and osteocytes. This is because the FDBB scaffold only uses a freeze-drying process.[10] On the otherhand the dc-FDBB scaffold, before the freeze-drying process was carried out on the dc-FDBB scaffold, a decellularization process was carried out first using a 0.5% SDS solution for 24 hours. SDS solution is an ionic surfactant whose action tends to be stronger than other types of surfactant solutions.[17,18] The way SDS works is to dissolve the cytoplasm and nuclear membrane. It can reduce more than 90% of cell DNA in the tissue.[19] In the DBBM scaffold, a combustion process was carried out (furnize) at a temperature of 1000°C to remove protein components and remain inorganic components in the form of minerals then rinsed again with sterile distilled water and dried in an oven until the moisture content is below 10%. Thus protein components including bone graft material cells were lost after processing was carried out.[11]

It should be highlighted that decellularization could affect the composition of the extracellular matrix and cause structural disturbances. The goal of the decellularization

process is to minimize side effects. The use of SDS can remove Glycosaminoglycans (GAGs) therefore loss of water content and affect the matrix structure. The biomechanical properties of the extracellular matrix after decellularization are always lower than beginning.[20] The results of research showed the differences in the structure of the extracellular matrix between the FDBB and dc-FDBB scaffolds.[21] Extracellular matrix structure dc-FDBB scaffold was degradation meanwhile in FDBB scaffold a good extracellular matrix structure was needed to achieve an osteoconductive scaffold.

Histological image of the DBBM scaffold was not absorbed HE staining. On the other hand it was difference with two groups of scaffolds. This was because the DBBM scaffold does not have organic or protein components. Therefore, there was no affinity between the tissue and the HE staining. In the FDBB and dc-FDBB scaffold groups, the HE staining was well absorbed because the FDBB and dc-FDBB scaffolds still had organic components and proteins that could bind to HE stainings.[20]

According to Crapo et al. (2011), the criteria for bone graft material that is sufficient to meet the criteria if bone graft material: (i) <50 ng DNA per mg extracellular matrix dry weight, (ii) <200 bp DNA fragments length, (iii) lack of visible nuclear material in tissue sections stained with Hematoxylin Eosin (HE) or 4',6-Diamidino-2-Phenylindole (DAPI). From the three criterias, this study can determine the first criteria through DNA quantification and the third criteria by HE staining. DNA quantification presented that the highest average DNA concentration was found on the FDBB scaffold (19.75 ng/μL), followed by the dc-FDBB scaffold (16.84 ng/μL) and the DBBM scaffold (8.72 ng/μL). The DNA concentration of the FDBB scaffold was the highest among of the dc-FDBB and DBBM scaffolds. There were no significant difference between the FDBB and dc-FDBB scaffold groups, but there was a significant difference between the FDBB and DBBM scaffold groups, and the dc-FDBB and DBBM scaffold groups. This happened because the FDBB and dc-FDBB scaffold groups contained higher concentrations of DNA than the DBBM group. The decellularization process carried out on FDBB was not effective in lower antigenic potential.[21]

According to Shen (2019), DNA purity test is considered ideal with ratio of A260/280 scoring ≥1.8 with maximum of 2.0. Ratio below 1.8 revealed protein contamination on DNA samples. In this study, mean DNA purity values were 1.60, 1.63, 1.48 for FDBB, dc-FDBB, and DBBM, respectively. Thus, DNA concentration measurement results on the three scaffolds did not achieve good quality as a result of protein contamination. It is expected that in the next research the value of DNA purity can reach the purity value of the A260/A280 ratio in the range of 1.8-2.0.

Conclusions

It is concluded that, FDBB and dc-FDBB scaffolds have not antigenic potential. Further in vitro, as well as in vivo studies are required to confirm its antigenic potential.

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Ethical policy and institutional review board statement

Ethical clearance had been obtained from the Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga Surabaya (629/HRECC.FODM/XII/2021), 2021.

Declaration of Interest

The authors of this manuscript declare no conflicts of interest in this article.

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