Cell Attachment and Biocompatibility Analysis of Freeze-Dried Bovine Bone Scaffold and Decellularized Freeze-Dried Bovine Bone Scaffold on Human-Umbilical Cord Mesenchymal Stem Culture

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Abstract

Freeze-Dried Bovine Bone Scaffold (FDBB) and Decellularized Freeze-Dried Bovine Bone (DC-FDBB) are promising new alternative of xenograft material in bone tissue engineering. However, their biocompatibility is still unknown.

To investigate whether FDBB and DC-FDBB scaffolds are biocompatible, able to induce cell proliferation attachment in vitro.

Human umbilical cord mesenchymal stem cells (hUC-MSCs) culture was exposed to FDBB, DC-FDBB, Deproteinized Bovine Bone Matrix (DBBM) scaffold (as positive control) conditioned medium for 24, 48 and 72 hours (n=8). MTT assay was then performed to measure the number of viable cells at each observation time. Normality (Shapiro-Wilk) and homogeneity (Levene) test were done on the results of the MTT assay, then ANOVA test was performed. To support the finding, further SEM observation was performed. hUC-MSCs were seeded on the surface of each scaffold type (n=3) and incubated for 24, 48 and 72 hours, then SEM observation was done on the scaffold surface to analyse their surface cell attachment.

The mean percentage of living cells in FDBB group was the highest among all groups. There were significant differences ($p<0.05$) in each treatment group at each observation time, except between FDBB and DC-FDBB (48 hours), DC-FDBB and DBBM (48 hours), FDBB and DC-FDBB (72 hours) ($p>0.05$). SEM examination showed the same results, as the highest number of cell colonization was found on FDBB group at each observation time.

FDBB and DC-FDBB scaffolds have good biocompatibility characteristics that can be used as a bone substitute in bone tissue engineering.

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Introduction

There are several conditions that may cause the bone not be able to heal spontaneously without any surgical reconstructive intervention, for example in critical size defect. Critical size defect occurs when the size of the defect is more than 1-2 cm width or more than 50% of the bone circumferential.[1,2,3] Scaffold is needed to reconstruct the critical size defect in the jawbone. The scaffold functions as a 3-dimensional (3D) framework for cell attachment (osteoconduction) so that cells can grow, proliferate, and differentiate into osteoblastic line cells (osteoinduction), and form new bone.[4,5]

Xenomaterial scaffolds derived from bovine bone have been developed as an alternative. Bovine bone has a relatively larger size so that they are more flexible to be shaped into a certain size. In addition, the availability of bovine bone is much higher than allograft and autogenous bone graft. Bovine bone scaffold has

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osteoconductive and osteoinductive properties because its extracellular matrix (ECM) contains various components, such as: type I collagen, type II collagen, non-collagenous proteins (glycosaminoglycans, chondroadherins, and Bone Morphogenic Proteins (BMPs)) that support osteoinductive characteristics. Based on the manufacturing process, there are 3 variants of bovine bone scaffold, namely: deproteinized bovine bone matrix (DBBM), frozen dried bovine bone (FDBB), and demineralized frozen dried bovine bone (DFBB).[6]

Demineralized Bovine Bone Matrix (DBBM), also known as Bovine hydroxyapatite (BHA), is a bone scaffold material that is often used clinically. Some brands that have been widely circulated in the market are Bio-Oss® and GamaCHA®. However, during manufacturing process, residual cells along with protein components from the ECM will be lost due to the combustion process at a temperature of 1000°C.[7] This process will reduce the immunogenic potential of the scaffold so that it has good biocompatibility, but will reduce its osteoinductive ability. The osteoconductive property is still present due to the porosity and interconnection of the hydroxyapatite crystal structure of DBBM.[8,9] FDBB was chosen as an alternative to DBBM because it contains ECM components, so it still has osteoinduction and osteoconduction properties. However, it is still possible that FDBB contains DNA components, cell residuals, and native proteins that may induce immune and inflammatory responses thus, cause cell toxicity.[10,11]

The biocompatibility of a biomaterial is important because it shows the ability of a material to interact with living cells or tissues or metabolic systems.[12] The toxicity of a substance can be measured based on the level of viability and/or the rate of cell proliferation, one of which is by using the Micro tetrazolium (MTT) Assay.[13] In this study, the *in vitro* biocompatibility between FDBB, DC-FDBB and DBBM will be compared through a cytotoxicity test to describe the biological reaction between the two materials and cells through the MTT assay in human umbilical cord mesenchymal stem cell (hUC-MSCs) cultures. Furthermore, the description of the pattern of cell attachment on each scaffold will be evaluated using a Scanning Electron Microscope (SEM).

Materials and methods

The Universitas Airlangga, Faculty of Dental Medicine ethics committee granted ethical approval for this *in vitro* hUC-MSCs culture experiment (150/HRECC.FODM/IC.2021)

This true-experimental research was done on *in vitro* hUC-MSCs culture. Bovine scaffold was divided into 3 treatment groups based on the method of manufacture, namely FDBB, DC-FDBB, and DBBM, then a conditioned medium scaffold was made for each treatment group. MTT assay was done on hUC-MSCs cultures which exposed to the scaffold conditioned medium. The assay was carried out at 3 observation times (24, 48, and 72 hours; n=8) to observe the mean percentage of living cells. In addition, SEM observation on scaffold surface was done at the same observation times (n=3).

Scaffold Manufacture

All scaffolds were made of cancellous diaphysis of bovine femur. Based on scaffold manufacturing protocol by Installation of Cell and Tissue Bank, RSUD Dr. Soetomo, Surabaya, Indonesia, to make FDBB scaffolds, firstly, the bone was cut into 10 x 5 x 5 mm blocks, then put into 3% hydrogen peroxide to clean blood, fat, and bone marrow remnants. After rinsed with sterile aquadest, the bone blocks were frozen in the temperature of 80°C and dried with lyophylizer until the water content reached below 10%. Then, scaffolds were packed and sterilized using gamma ray radiation. DC-FDBB scaffolds were made using the same method, but DC-FDBB scaffolds underwent further rinsing with sodium dodecyl sulfate (SDS) 0.5% before freeze-drying process. DBBM scaffolds were also made using the same method as FDBB scaffolds, but without the freeze-drying process. Instead, 1000°C furnace process was performed to eliminate protein component while preserving mineral content before scaffold packing and gamma-ray sterilization.

Conditioned Medium Preparation

Conditioned medium was made using a modified method from Filho et al. Each scaffold was immersed in Alpha Modification of minimum essential medium eagle (α-MEM), penicillin/streptomycin 100 U, dan L-Glutamine 2mM with the ratio of 1 g scaffold: 10mL culture medium (10% w/v) for 48 hours. Then, it was centrifuged (600 gf, 20°C for 8 minutes) and the supernatant was filtered (0.22 mM).[14]

Culture hUC-MSCs Preparation

Umbilical cord sample-taking was performed using a method from Hendrijantini et al. 3-5 cm of umbilical cord was cut and rinsed with Phosphate Buffer Saline (PBS) 3 times to rinse blood remnants, then immersed in a solution of ringer lactate, gentamycin 2.5 µg/mL and amphotericin 1000 U/mL for 20 minutes. Warthon's jelly was separated from umbilical artery and vein, then the remaining umbilical cord was cut into 1 mm3 fragments and undergone enzymatic digestion with Phosphate Buffer Saline (PBS), 0.75 mg/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.075 mg/ml DNAase I (Takara Bio, Shiga, Japan). After incubated at 37^{o}C and centrifuged with magnetic stirrer for 10 minutes, the solution was filtered using 100 µm cell strainer. The cells were cultured with the density of 1 x 10^5 cell/cm^2 and put into culture flasks (growth medium: Alpha Modification of minimum essential medium eagle (α-MEM) and fetal bovine serum (FBS) 20% (Gibco BRL, Gaithersburg, MD, USA), with Epidermal Growth Factor (EGF) 10ng/mL, penicillin/streptomycin 100 U, and L-Glutamine 2mM supplementation. The culture medium was changed every 3 days until 80% cell confluence was reached. hUC-MSCs were validated using Cluster Differentiation (CD)105, CD90, CD73 positive markers and CD34 negative markers (**Figure 1**). After trypsinization with 0,05% trypsin EDTA was done, the cells were further cultured in 60 or 80 mm tissue culture dishes (corning) until the fifth passage as needed in this study.[15]

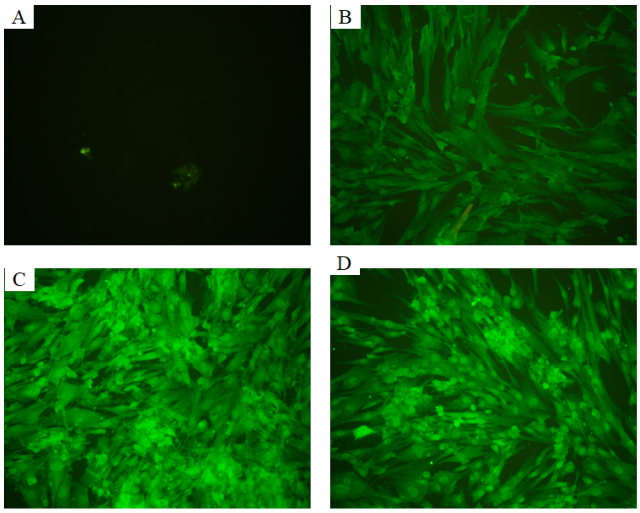

Figure 1: Immunofluoresence of hUC-MSCs Shows: A. Negative CD34, B. Positive CD73, C. Positive CD90, D. Positive CD105.

Cytotoxicity Test (MTT Assay)

The proliferation and viability of hUC-MSCs were measured using a modified Chen et al MTT assay. Briefly, cells were cultured in three 96-well plates at an initial density of 5 × 10^3 cells / 150 µl per well. At each plate, cells were divided into 3 groups: FDBB, DC-FDBB, DBBM (as positive control), while using growth medium (α-MEM + L-Glutamine 2mM, antibiotic, antifungal) as negative control (n=8). Following the 24 hours incubation time (37^{O}C, 98% humidity, and 5% CO_2), 145 µL medium was removed. Then, a mixture of 10% FBS (Gibco BRL, Gaithersburg, MD, USA) and FDBB (for FDBB group), DC-FDBB (for DC-FDBB group), and DBBM (for DBBM group) conditioned medium was added into each treatment group plates, and incubated according to observation time (24, 48 and 72 hours). Following the incubation time, 25 µl/well of MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and then, the plates were incubated at 37˚C for 4 hours again. The formazan crystals were solved by adding 50 µl of DMSO solution (Sigma-Aldrich, St. Louis, MO, USA). After mixing them thoroughly, the absorbance values were determined using ELISA reader at a wavelength of 595 nm.[16]

Scaffold Surface and Cell Attachment Observation

The procedure was done based on the protocol at Institute Biology and Science Universitas Brawijaya, Malang, Indonesia. The solution of conditioned medium and scaffold was fixated with *glutaraldehyde* 2,5% for 2-3 hours, then rinsed with running water for 30 minutes and immersed gradually in 50-90% alcohol for 90 minutes, respectively, then in absolute alcohol for 3x90 minutes or overnight. The samples were dried into desiccator for 4x90 minutes or overnight. The dried samples were put on cover glass, then coated using Q15RS Platinum Coating (Quorum Laughton, UK). Then, the samples were observed using Scanning Electron Microscope (SEM) Hitachi TM-3000 (Hitachi, Minato-ku Tokyo, Japan). The digital data of SEM characterization was analyzed using Hitachi TM-3000 Software (Hitachi, Minato-ku Tokyo, Japan) to determine seeded cell distribution.

Statistical Analysis

After testing data normality (Shapiro-Wilk's test) and homogeneity (Levene's test) ($p>0.05$) on the results of the MTT assay,

ANOVA test was carried out to compare the data then continued with post hoc test Bonferroni ($p<0.05$). Cell morphology on the scaffold surface of each treatment group and at each observation time was then observed using SEM (n=3).

Results

Normality test (Shapiro-wilk's) showed normal distribution on all groups ($p>0.05$) except on 24 hours control group ($p = 0.027$). All data are homogen based on Levene's homogeneity test (Table 1). Overall, although there was a significant increase in cell proliferation rate at 24, 48, and 72 hours in each treatment group, the mean percentage of living cells in the FDBB group was the highest compared to the other two groups **(Table 1 and 2).**

Table 1. Normality and homogeneity test results.

	Observation time (hours)	Mean ± SD	Normality (p)	Homogeneity (p)
FDBB	24	0.268 ± 0.033	0.434	0.39
	48	0.334 ± 0.015	0.552	
	72	0.369 ± 0.038	0.756	
DC-FDBB	24	0.258 ± 0.039	0.683	0.16
	48	0.318 ± 0.014	0.465	
	72	0.396 ± 0.015	0.756	
DBBM	24	0.237 ± 0.185	0.102	0.278
	48	0.294 ± 0.024	0.323	
	72	0.341 ± 0.012	0.146	
Negative control group	24	0.370 ± 0.060	0.027*	0.120
	48	0.448 ± 0.031	0.898	
	72	0.498 ± 0.030	0.183	

Note: *information: non-homogenity data ($p<0.05$).

Table 2. Percentage of living hUC-MSCs after scaffold conditioned medium exposure.

	24 hours	48 hours	72 hours
FDBB	65.8%	70.9%	75.9%
DC-FDBB	62.4%	66.9%	70.3%
DBBM	55.3%	60.6%	63.0%

ANOVA and Kruskal-Wallis tests showed that there was significant difference on the average of living hUC-MSCs between each treatment group. Bonferroni test was done to further compare the difference between each group **(Table 3 and 4).**

	Observation time (hours)		Significancy (p)
FDBB	24	48	0.001*
		72	0.001*
	48	72	0.001*
DC-FDBB	24	48	0.001*
		72	0.001*
	48	72	0.001*
DBBM	24	48	0.001*
		72	0.001*
	48	72	0.001*

Note: *information: significant different at $p<0.05$.

Table 3. Bonferroni test results according to observation time.

The number of viability percentage of hUC-MSCs significantly increased at each observation time in each scaffold type **(Table 3).** There were significant differences ($p<0.05$) among each treatment group at each observation time, except for the FDBB and DC-FDBB groups (48 hours), DC-FDBB and DBBM (48 hours), FDBB and DC-FDBB (72 hours) **(Table 4).**

Observation time	Scaffold types		Significancy (p)
24 hour	FDBB	DC-FDBB	0.001*
		DBBM	0.001*
		Control	0.001*
	DC-FDBB	DBBM	0.001*
		Control	0.001*
	DBBM	Control	0.001*
48 hour	FDBB	DC-FDBB	1
		DBBM	0.01*
		Control	0.001*
	DC-FDBB	DBBM	0.27
		Control	0.001*
	DBBM	Control	0.001*
72 hour	FDBB	DC-FDBB	1
		DBBM	0.001*
		Control	0.001*
	DC-FDBB	DBBM	0.001*
		Control	0.001*
	DBBM	Cont	0.001*

Note: *information: significant different at $p<0.05$.

Table 4. Bonferroni test results according to scaffold types.

Based on SEM observation findings, all scaffolds showed different surface morphology (Figure 2). At 100x magnification, FDBB scaffold had 214-423 nm porosity diameter, while DC-FDBB scaffold 169–597 nm and DBBM scaffold 126-589 nm. Cracks were seen on DBBM scaffold surface (50x magnification).

The results of SEM observation also support our MTT-assay finding, namely the most colonization of cells was found in the FDBB group at each observation time compared to the DC-FDBB and DBBM groups. However, cell

colonization was seen on all scaffold surface and its number was increased following each observation time (24, 48, and 72 hours) (Figure 3-5).

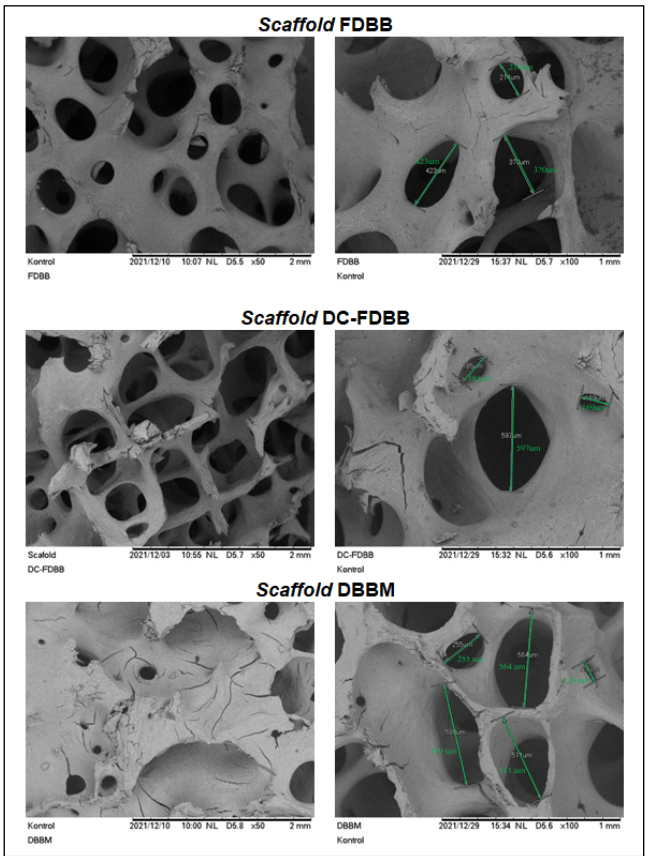

Figure 2: Surface morphology of FDBB, DCFDBB, and DBBM scaffold with 50 and 100 x magnification.

mechanical properties, this method allows to produce scaffold that not only can be easily stored and have osteoinductive properties, but also more resist- ant to radiation exposure that is routinely used to sterilize the scaffold.[19,20,21]

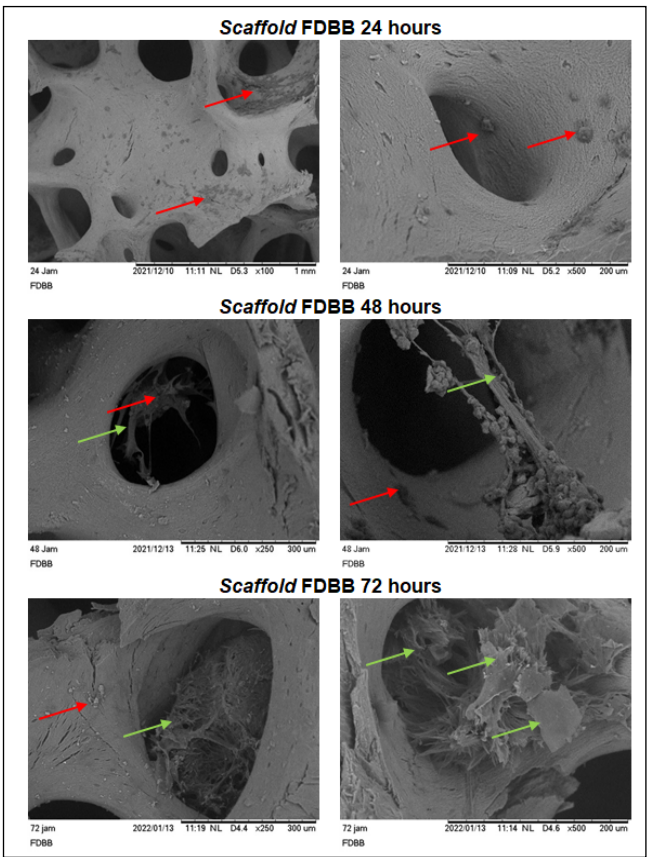

Figure 3: Image of cell colonies (red arrows) and cells that have formed ECM (green arrows) on the surface of the FDBB scaffold with treatment times of 24, 48, and 72 hours (250 and 500 x magnification).

Discussion

Ideally, scaffold materials should mimic the characteristics of natural bone that able to create suitable biochemical environment and provide biomechanical support for cell adhesion, migration, proliferation, osteogenic differentiation, and angiogenesis in the scaffold.[17] Based on the manufacturing process, there are 3 kinds of bovine bone scaffold, namely: FDBB, DFDBB, and DBBM.[6] To reduce their immunogenicity and the risk of disease transmission, freeze-drying process was used to manufacture both FDBB and DC-FDBB scaffolds. During the freeze-drying process, the bone is firstly frozen, then dried using atmospheric pressure to vaporize its water content (water sublimation) without any condensation.[18] Although it alters the scaffold's

In this research, the scaffold was made from cancellous diaphysis bovine femur. It was cut into blocks, then chemically washed to clean the remaining blood, fat and bone marrow using non-ionic hydrogen peroxide 3%. After rinsing with sterile distilled water to clean the remaining peroxide solution, freeze drying process was conducted. While FDBB scaffold was made using the above mentioned method, DC-FDBB scaffold went through additional washing process with ionic detergent (SDS 0.5%) before freeze-dried to remove the remaining cell components and preserve molecular contents and extracellular matrix rigidity.[10]

On the other hand, DBBM, also known as bovine hydroxyapatite (BHA), is a bone scaffold

material that is often clinically used. Unlike FDBB and DC-FDBB scaffold, DBBM scaffold does not undergo freeze-drying process. After being rinsed with aquadest, DBBM scaffold was heated at a temperature of 1000°C to remove protein components and preserve mineral components. As a result, residual cells along with protein components from the extracellular matrix (ECM) will be lost.[7] This process causes the reduction of immunogenic potential of the scaffold so that it has good biocompatibility, but loses its osteoinductive ability.

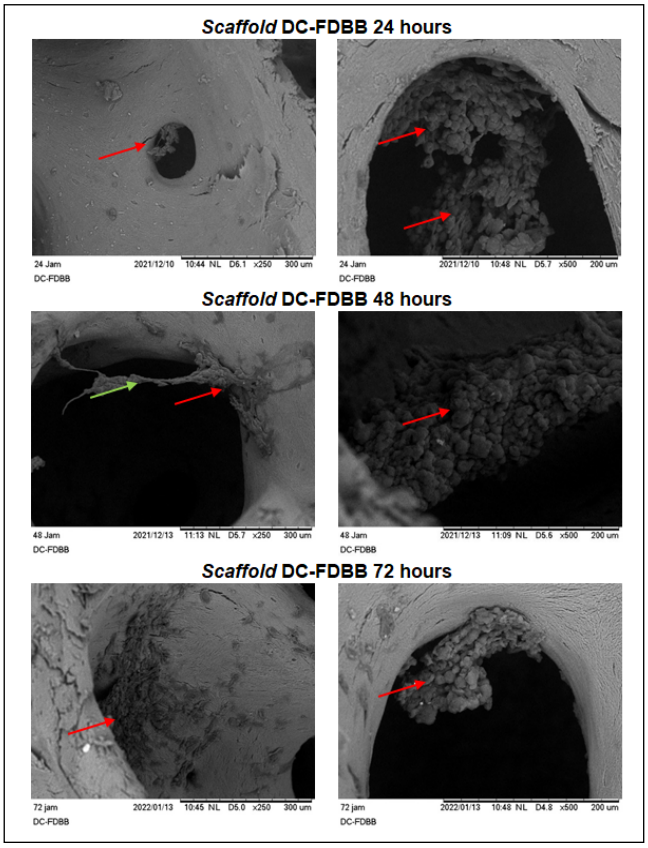

Figure 4: Image of cell colonies (red arrows) and cells that have formed ECM (green arrows) on the surface of the DC-FDBB scaffold with treatment times of 24, 48, and 72 hours (250 and 500 x magnification).

As FDBB scaffold still contains ECM components, its osteoinductive and osteoconductive properties was proposed to be better than DBBM scaffold so that it can be chosen as an alternative substitute. However, FDBB may contain DNA components, cell residuals, and native proteins that are able to induce immune and inflammatory responses, and cause cell toxicity. In addition, they also can be induced by the byproducts of scaffold manufacturing process, such as detergent.[10,11]

Based on the results of this study, all scaffolds (FDBB, DC-FDBB and DBBM) may not have cytotoxicity characteristic, as the mean percentage of viable cells were above 50% in all groups. Besides, the mean percentage of viable cells in FDBB and DC-FDBB groups was significantly higher than in the DBBM group ($p<0.05$). These results were obtained at each observation time (24, 48, and 72 hours). The result of this study is in line with a research by Lesmaya, et al which used granulated frozen bovine bone. Qualitative observations using SEM were also carried out to support our MTT assay results. In line with MTT assay results, it was observed that cell colonization occurred the most in FDBB group at each observation times while the least DBBM group.[22]

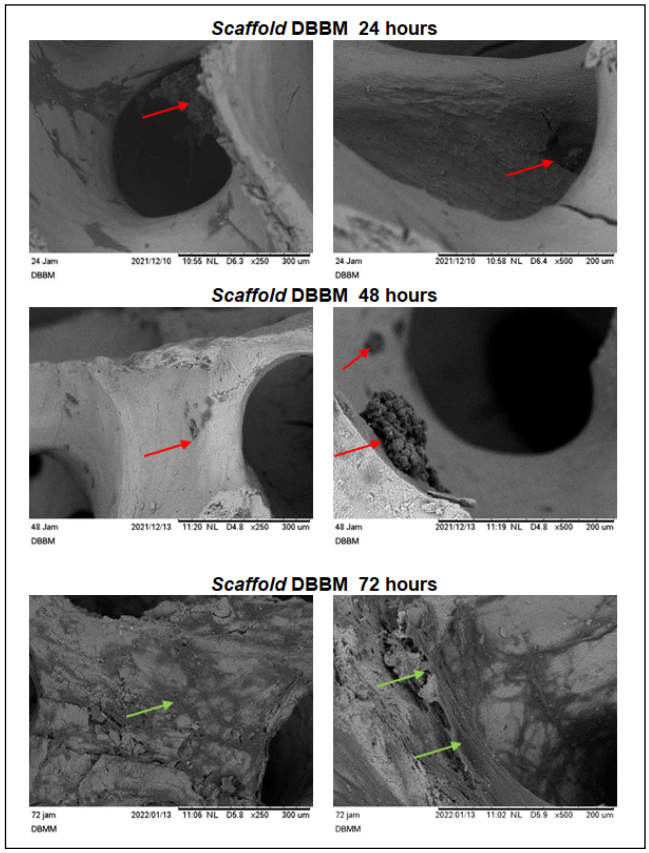

Figure 5: Image of cell colonies (red arrows) and cells that have formed ECM (green arrows) on the surface of the DBBM scaffold with treatment times of 24, 48, and 72 hours (250 and 500 x magnification).

This may be due to the presence of extracellular organics in the ECM of FDBB and

DC-FDBB scaffold. Recent research by Lin *et al.*, stated that ECM is involved in regulating cell adhesion, proliferation, response to growth factors, differentiation, and functional characteristics of adult bone.[23] ECM also provides excellent biochemical environment and biomechanical signals to initiate cell migration, differentiation, morphogenesis, and homeostasis.[24] The presence of organic components in FDBB and DC-FDBB scaffold ECM, plays an important role in inducing bone formation.[25] Organic ECM consists of type I collagen (90%), and noncollagenous proteins (10%). Non-collagenous proteins can be classified into: g-carboxyglutamate-containing proteins, proteoglycans, glycoproteins, and N-linked small integrin-binding ligands (SIBLIN).[26,27] Besides, according to Meredith, et al, ECM also plays an important role in cell survival, as it can prevent the expression of genes that regulate cell death program through the mediation of integrins.[28]

Although DBBM scaffold does not have ECM, it still can be used routinely as scaffold material as it is not toxic and still has osteoconductive properties due to its surface porosity and interconnection of the hydroxyapatite crystal.[8,9] Calcium (Ca^{2+}) and phosphate ($PO4^{3-}$) concentration in bovine bone may increase cell affinity through ion exchange with surrounding extracellular fluid. These ions penetrate the hydrophobic membrane of cell, which coated by cytoplasm skeleton. Later, the skeleton creates organelles, such as cilia or filopodia, which will expand and make an adhesion on hydroxyapatite surface.[29,30] FDBB and DC-FDBB contain not only hydroxyapatite crystal but also ECM, so that they can stimulate better cell adhesion and further cell proliferation and differentiation.

The mean percentage of living cells and observed cell colonization in the FDBB group were slightly higher than those in the DC-FDBB group at all observation times ($p>0.05$). It is possible that SDS removes some ECM components of DC-FDBB scaffold. SDS 5% is an ionic detergent that can interact with cell membranes, including plasma and nuclear membranes. It can cause cell lysis and destroy ECM proteins by re-folding protein molecules.[31,32] However, further studies are needed on the effect of SDS on scaffold characteristics and its ECM composition.

Conclusions

Freeze-dried bovine bone scaffolds and decellularized freeze-dried bovine bone scaffolds within limitation of the study have biocompatibility characteristics (*in vitro*) that that make them potential candidates for tissue engineering scaffold

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Declaration of Interest

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

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