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Research Report

Cytotoxicity test and characteristics of demineralized dentin matrix scaffolds in adipose-derived mesenchymal stem cells of rats

Desi Sandra Sari,[1] Ernie Maduratna,[2] F. Ferdiansyah,[3,5] I Ketut Sudiana,[4] and **Fedik Abdul Rantam[5,6]**
[1]Department of Periodontics, Faculty of Dentistry, Universitas Jember, Jember – Indonesia
[2]Department of Periodontology, Faculty of Dental Medicine, Universitas Airlangga, Surabaya – Indonesia
[3]Department of Orthopedics & Traumatology, Dr. Soetomo General Hospital, Surabaya – Indonesia
[4]Department of Electron Microscopy, Faculty of Medicine, Universitas Airlangga, Surabaya – Indonesia
[5]Regenerative Medicine and Stem Cell Centre, Universitas Airlangga and Dr. Soetomo General Hospital, Surabaya – Indonesia
[6]Stem Cell Laboratory, Stem Cell Research and Development Center, Universitas Airlangga, Surabaya – Indonesia

ABSTRACT

Background: *Demineralized dentin matrix (DDM) scaffold is a substitute material for the bone contained in human teeth. DDM is a scaffold-derived tooth dentine containing type I collagen and bone morphogenetic protein (BMP). While DDM possesses the ability to perform osteoinductive and osteoconductive roles, a cytotoxicity test of DDM scaffold remains extremely important in evaluating the level of toxicity of a material if cultured in cells. Adipose-derived mesenchymal stem cells (ADMSCs) are multipotent in nature because they contain progenitor cells and have the potential for differentiation via adipogenic, osteogenic and chondrogenic pathways. ADMSCs are also known to have high biocompatibility and the ability to combine with other bone material.* ***Purpose:*** *The purpose of this study was to determine the cytotoxicity and characteristics of DDM scaffolds derived from bovine teeth in the ADMSCs of rats cultured in vitro.* ***Methods:*** *This research constituted an experimental study. ADMSCs were isolated from the inguinal fat of rats. Thereafter, DDM was extracted from bovine teeth and formed 355-710 µm-sized particles. DDM scaffolds were assessed using SEM and the effects of DDM scaffolds on the cell viability of ADMSCs at concentrations of 10%, 50%, and 100% analyzed by means of 3-4,5'dimethylihiazol-2-yl,2.5-di-phenyl-tetrazolium bromide (MTT) assay. The results obtained were then analyzed by an ANOVA to establish the difference between the groups.* ***Results:*** *SEM results showed the diameter sizes of the dental tubulis DDM scaffolds to be approximately 4.429 µm and 7.519 µm. The highest cell viability (97.08%) was found by means of an MTT test to be in ADMSCs at a concentration of 10% compared to those at concentrations of 50% and 100%.* ***Conclusion:*** *In conclusion, DDM scaffold derived from bovine teeth with a particle size of 355-710 µm produces a low cytotoxicity effect on ADMSCs.*

Keywords: *adipose-derived mesenchymal stem cells; cytotoxicity test; demineralized dentin matrix; scaffold*

Correspondence: Desi Sandra Sari, Department of Periodontics, Faculty of Dentistry, Universitas Jember, Jl. Kalimantan 37 Jember 68121, Indonesia. E-mail: desi_sari.fkg@unej.ac.id

INTRODUCTION

Demineralized dentin matrix (DDM) derived from bovine teeth is a material used as a substitute for human teeth. In recent years, DDM has widely been used in dental research as it is easily obtained in large quantities of high quality with a more uniform composition than that of human teeth.[1,2]

Bovine dentin is similar in composition to human dentin which consists of 70% inorganic material, 20% organic material and 10% water. Dentine microstructure consists of collagen fiber tissue[3] and contains various growth factors, such as insulin growth factor-2 (IGF-2), bone morphogenetic protein (BMP), tumor growth factor-ß (TGF-ß), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF).[2,4,5]

DDM can play an important role as scaffold because it contains both organic and inorganic components in addition to being microporous.[6] Ideally, scaffold must be biocompatible, biodegradable and non-toxic. Moreover,

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DDM also possesses the ability to proliferate and differentiate between cells and is, therefore, considered a scaffold deployable as a bone substitute.[7,8]

According to the findings of several studies, DDM is not only biocompatible, but also executes osteoinductive and osteoconductive roles.[9,10] Nevertheless, demineralized bone and dentin can decrease its antigenic properties.[11] The osteoconductive activities of scaffolds can provide a microenvironment supportive of new bone growth. On the other hand, its osteoinductive activities involve growth factors as well as adhesion of exogenous and endogenous progenitor cells to proliferate, differentiate and produce bone cells.[12,13]

Adipose-derived mesenchymal stem cells (ADMSCs) demonstrate a multi-differentiational ability because they contain numerous multipotent progenitor cells and possess differentiation abilities by means of adipogenetic, osteogenetic and condrogenetic pathways.[14] Therefore, when combined with the appropriate scaffold ADMSCs are expected to improve the healing process of bone damage in the maxilla, mandible and calvarium. The combination of ADMSCs and inorganic bovine bone can even stimulate proliferation and differentiation within osteogenics.[15] The viability of hydroxyapatite (HA) derived from tooth bone indicates that this material is non-toxic when cultured in bone marrow mesenchymal stem cells and can be used as an alternative bone graph material.[16] Hence, this study aimed to determine the cytotoxic characteristics and effects of 355-710 µm-sized DDM scaffolds derived from tooth bone on the ADMSCs of rats using an in vitro technique.

MATERIALS AND METHODS

This research was approved by the Research Ethics Committee, Faculty of Veterinary Medicine, Universitas Airlangga (number 637-KE). Three 4-week old, male, Wistar rats were sacrificed to obtain fat tissue from the perirenal region. In order to isolate the ADMSCs, their adipose tissue was washed with phosphat-buffer saline (PBS) containing 10% penicillin-streptomycin mixture (Sigma-Aldrich, USA). Meanwhile, their fat tissue was chopped into small pieces, soaked in 0.2% type I collagenase (Worthington, USA), added to PBS and agitated slowly for 40 minutes at 37^0C. It was then filtered with a 10 µm mesh (SPL, Korea) before being centrifuged at 1,250 rpm for five minutes to remove the supernatant.[17,18]

The ADMSCs were cultured with Eagle Alpha-modified minimum essential medium (α-MEM) (Gibco, USA) mixed with 15% fetal bovine serum (Biowest, USA), 2 mm of L-glutamine (Sigma-Aldrich, USA), 100 IU/ml of penicillin (Sigma-Aldrich, USA), 100 mg/ml streptomycin (Sigma, USA), and 2.5 µg/ml of fungizone (Sigma-Aldrich, USA) and subsequently incubated at 37^0C with 5% CO_2. The cells were grown in six wells on tissue culture plates (Iwaki, Japan) at a concentration of 10^7 in each well. Observations were then completed with an inverted microscope at 80x magnification. In cases of nucleus cells that were similar to fibroblast cells, their morphology was evaluated, while their attachment to the plastic culture plates was passaged 4-5 times.[19,20]

Characterization of ADMSCs in the culture media was carried out by means of an immunocytochemical staining technique. Single cells derived from the trypsinization process were centrifuged. The pellets and medium were then re-suspended and placed on glass objects, before being incubated at 37^0 C for one hour. They were subsequently fixated with formaldehyde (Bioworld, USA), and washed with PBS. Samples plus anti-rat CD 105 FITC (Biolegend, USA) and anti-rat CD 45 FITC (Biolegend, USA) were incubated 37^0C for 45 minutes.[17,18]

The production and processing of scaffolds derived from bovine teeth were conducted at the Tissue Bank of Dr. Soetomo General Hospital. Bovine teeth were removed from the jaws of the subjects with osteotomes, hammers and saws. The teeth were cleaned by immersion in 3% peroxidase for one week and their crowns and roots were subsequently separated using a bone cutter and knable pliers. The process of producing a powder commenced with smashing the tooth roots, composed of dentine tissue and cementum, by inserted them into a bone miller. The resulting powder was sifted to produce particles of the desired size. The demineralization process, incorporating a bone mineral release method, was performed during which DDM particles were soaked in 1% hydrochloric acid (HCL) for one day, then washed thoroughly and dried.[21,22] Freeze-drying of DDM particles was conducted during which they were sublimated into a dry condition in the form of 355-710 µm-sized particles and sterilized at BATAN (Jakarta, Indonesia). The form of bovine tooth particles was tested by SEM (Central Laboratory, Universitas Negeri Malang).

A DDM toxicity test was carried out on ADMSC cell cultures during which DDM was immersed in *x*-MEM for 24 hours. The ADMSCs were centrifuged and the pellets cultured in 96 wells at a maximum of 5 x 10^4 cells per well, before being incubated for 24 hours at 37^0C with 5% CO_2. After 80% of the cells had developed, they were incubated for 20 hours at 37^0 C with 5% CO_2 before being added to 200 µl of the DDM supernatant. This had been immersed in *x*-MEM. 25 µl of 3-(4.5-dimethylthiazol-2YL)-2,5-diphenyltetrazolium bromide (MTT) (Bioassay system, USA) added to each well and incubated for four hours at 37^0 C. The results were subsequently observed under an inverted microscope. Color changes in the wells were then read by an Elisa reader at a wavelength of 595 nm.[16,23]

All data were expressed at the mean and SD The statistical analysis were performed using one-way ANOVA. P value <0.05 were considered statistically significant

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RESULTS

Observation conducted on the first day confirmed the appearance of prospective cells. After three days, the cell growth reached 80% confluence leading to the medium being changed. On the 15th day after phase 4, the cells filling the plates possessed a fibroblast-like shape. The ADMSCs culture results are contained in Figure 1.

The characteristics of the ADMSCs were observed through immunocytochemical examination to confirm whether those that had been cultured represented mesenchymal stem cells. Two markers of mesenchymal stem cells (MSCs) were obtained, namely; CD 105 as a positive marker and CD 45 as a negative marker. The immunocytochemical examination results showed a higher number of positive CD 105 markers than the negative CD 45 markers in passage 4 (Figure 2).

DDM scaffolds with a diameter of between 355 µm and 710 µm were produced at the Dr. Soetomo Hospital Tissue Bank in Surabaya. The results of micro-computed tomography (µ-CT) indicated that the differing diameters of the DDM scaffold particles rendered them heterogeneous. The distribution of particles was then measured from the medial axis. The results confirmed that the average size of DDM scaffold particles was within the range of 355-710 µm (Figure 3).

SEM images of DDM scaffolds indicated the various diameter sizes of the dentinal tubular pores. The smallest diameter was approximately 4.429 µm, while the largest was in the region of 7.519 µm (Figure 4).

The MTT assay performed produced significantly contrasting results between DDM scaffolds at concentrations of 10%, 50% and 100%. In DDM scaffold at 10% concentration, the average concentration of living ADMSCs was 97.08% with a mean ± SD value of 97.08 + 12.67. Meanwhile, in DDM scaffold at a concentration of 50%, the average number of living ADMSCs cells 88.58% with a mean ± SD value of 88.58 ± 12.38. Finally, in DDM scaffold at a concentration of 100%, the average number of living ADMSCs cells was 76.64% with a mean ± SD value of 76.64 + 5.76 as shown in Figure 5.

In addition, the formation of purple formazan crystals indicated that MTT would be reduced by the mitochondria in ADMSCs present in purple formazan compounds and insoluble in water. Hence, the higher the intensity of purple, the greater the number of cells that survive (Figure 6).

DISCUSSION

In this study, ADMSCs were derived from the adipose tissue of Wistar rats that had been passaged four times. During this process, the cells were attached to the plates and increased by approximately 80% with fibroblast-like morphologies (Figure 1). The growth of adipose MSCs can be sub-cultured up to 9-10 times before the cells degenerate. Research conducted by Yang et al. (2018) revealed that in vitro MSCs derived from bone marrow used for osteogenic purposes are supposed to be extracted in the fourth passage, rather than the eighth, since the former is considered to be the initial passage where cell communication is optimal and cell proliferation maximal. Therefore, the most widely used passages of ADMSCs culture in regeneration therapy are passages 1-5.[24,25]

Based on the results of this research, the particle sizes of DDM scaffolds obtained were between 355 µm and 710 µm (Figure 3), while the ideal particle size for bone graph material is 500 µm. This means that those obtained in this research were still recommended since resorption will continue for a significant period if the particle size is excessively large, while the particles will be absorbed before they can function as graft material if they are too small. In other words, the material particle size of bone graph can affect bone formation. Hence, small scaffold pore size can facilitate osteoblast cell proliferation, while low porosity can help in vitro osteogenic differentiation. Newly-formed bone structure is related to the pore size of the scaffolds in that smaller pores can support more trabecular formation.[26–28]

Based on the SEM analysis results, the DDM scaffold surface which was 4,429 µm and 7,519 µm in diameter with exposed dentinal tubules, dentine bundle bonds and intermittent peritubular capillaries provided a channel to release proteins and growth factors. Therefore, interconnecting porosity is the predominant factor in osteoconduction. However, a number of alternative arguments have been put forward stating that the pore size required for bone growth in implants is 100 to 500 µm.[29,30]

Several factors, such as the size of scaffold pores, must be considered when designing scaffolds since this will affect nutrient diffusion and cell migration throughout the scaffold, inhibit the formation of vascular tissue in the scaffold and facilitate integration with the surrounding vascular tissue. Previous research has gone as far as stating that collagen combined with demineralized bone powder with a particle size of 250-500 µm may prove suitable for osteoblast differentiation and, possibly, bone tissue engineering.[31,32]

The MTT assay indicate that DDM scaffolds at a concentration of 10% are non-toxic to ADMSCs cultures. The MTT assay is used to evaluate material cytotoxicity in tissue engineering as well as to provide an indication of cell growth and proliferation. The results showed that 10% DDM scaffold caused a lower incidence of ADMSC cell death than DDM scaffolds at concentrations of 50% and 100% ($p<0.05$) (Figure 5). This means that DDM scaffold is non-toxic and can be attached to mesenchymal stem cells derived from the adipose tissue of Wistar rats.[33]

It can be argued that a relationship exists between the cells and scaffold. Scaffolds can release a chemical messenger that binds to membrane receptors, subsequently affecting intracellular communication. Thereafter, growth factors will bind not only to cell membrane receptors

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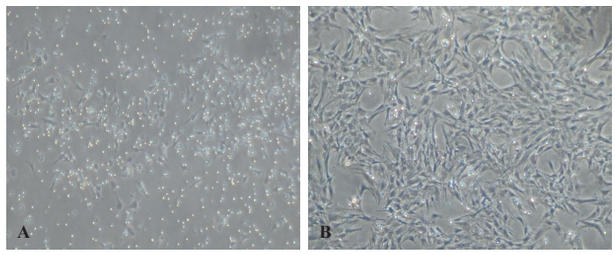

Figure 1. ADMSCs culture (A) Cells began to grow on the first day after the isolation, and cell colonies attached to the passage plate 1; (B) Cells became monolayer with fibroblast-like morphologies in passage 4 (with inverted microscope at 200x magnification).

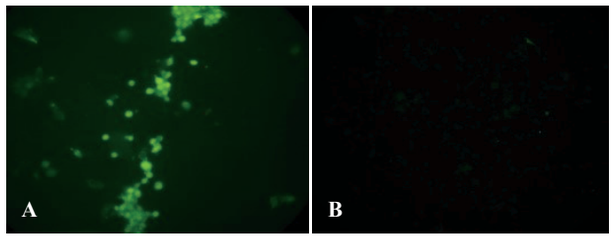

Figure 2. The immunocytochemical images of ADMSCs at the 4th passage (A) The expression of CD 105 markers looked strong with the presence of green fluorescent cells; (B) The expression of CD 45 markers looked weak marked with the absence of green fluorescent cells (with immunofluorence microscope at 200x magnification).

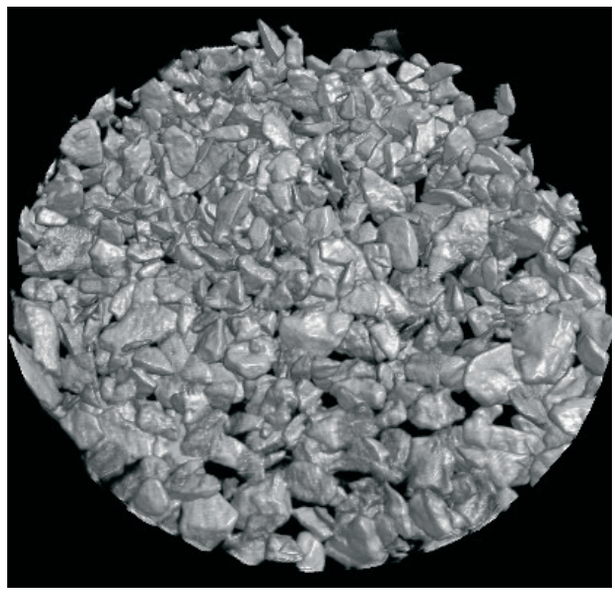

Figure 3. The 3D images of µ-CT showing the shape of DDM scaffold particles.

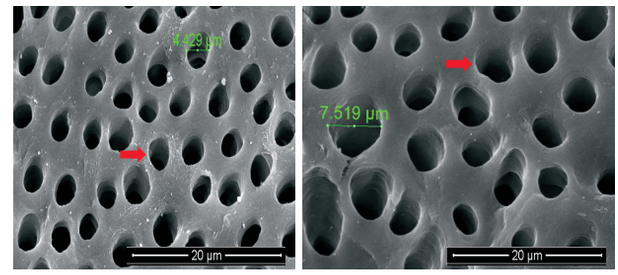

Figure 4. SEM images of DDM scaffolds depicting the presence of pores on the surface of the scaffolds (red arrows indicate dentinal tubular pores at 5000x magnification).

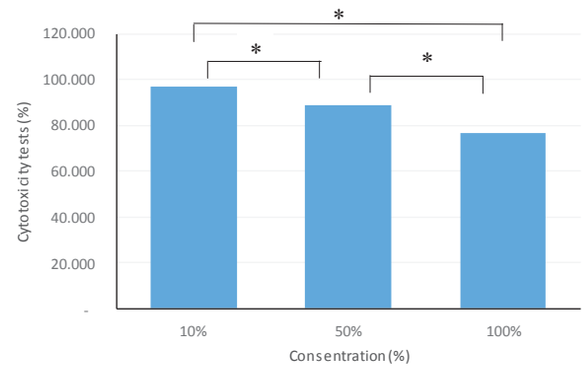

Figure 5. Results of the cytotoxicity tests on DDM scaffolds at different concentrations in ADMSCs cells (significance * p≤ 0.05).

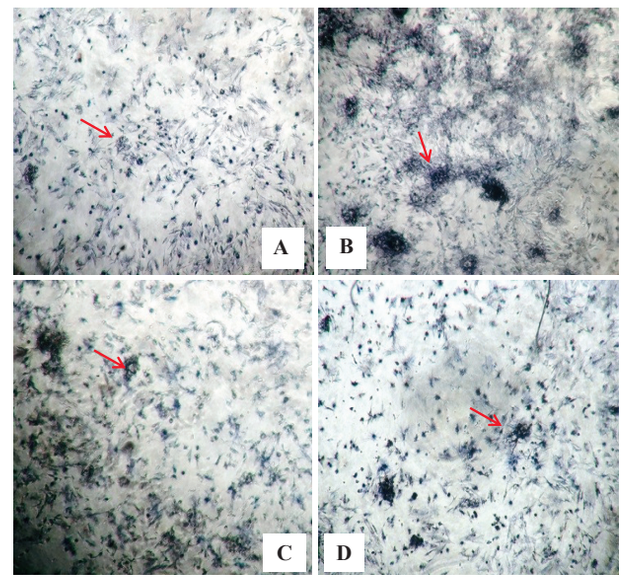

Figure 6. The formation of formazan crystals in which the cells became purple (see red arrows). (A) The control group; (B) MTT assay at a concentration of 10%; (C) MTT assay at a concentration of 50%; (D) MTT assay at a concentration of 100% (inverted microscope, 200x magnification).

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initiating the intercellular cascade that affects gene expression and molecular signals released from scaffolds, but also to cell receptors triggering cell intracellular communication. The link between DDM scaffolds and cells occurs through integrin molecules on the cell surface, where cell attachment to scaffold through integrins is very important for the migration, proliferation and differentiation of various cell types.[34,35] In conclusion, DDM scaffolds with a pore size of 355-710 µm derived from bone teeth will not produce cytotoxicity effects in ADMSCs.

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