iMEDiTEC 2017

ORIGINAL PAPER

Viability Bovine Tooth Hydroxiapatite on Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

Different types of bone-graft substitutes have been developed. Hydroxyapatite is a scaffold- has the ability to perform osteoinduction and osteoconduction activity. Of these, Bovine tooth hydroxyapatite (BTHA) is a novel material produced by Airlangga University. Bone marrow mesenchymal stem cells (BMSCs) is a potential material in the regenerative medicine. It has tissue source and multipotent somatic stem cells. BMSCs have been combined with biomaterials such as osteoinductive and osteoconductive scaffold in order to improve bone regeneration. This study was performed to determine the viability of BTHA on bone marrow stem cells. This study is an experimental research. BTHA was extracted from bovine's teeth and formed into particles 355-710 µm. The viability effect of BTHA scaffold on BMSCs in a variety concentration 10%,50% and 100% (w/v)were analyzed by using 3-(4,5'dimethylihiazol-2-yl),2.5-di-phenyl-tetrazolium bromide (MTT) assay. Results were analyzed with ANOVA to see the difference between group. The highest viability of the cells (97.1%)were found in concentration 10% by using MTT assay. Viability BTHA 10% and 50 % on BMSCs is not different significantly ($p \leq 0.05$). BTHA is viable in 10% and 50% on bone marrow mesenchymal stem cell. In addition, economical production of BTHA should be taken into consideration. In future, BTHA may be a viable alternative for bone grafting when clinical trials have been completed.

INTRODUCTION

Periodontal disease is one of the dental and oral health problems that still has a high prevalence in people, where periodontal disease in all age groups in 2013 in Indonesia is 96.85% (Balitbang Kemenkes RI, 2013). Surgical procedures in current periodontal disease have largely focused on the effects of bone defects as well as hard and soft tissues. Ideally the success of a treatment is expected to occur new bone regeneration, cementum and attachment of the periodontal ligament to replace the lost. To obtain these results, various materials and regenerative procedures have been used. Many of today's procedures use bone graft and bone substitutes (Stuart, Mea, Dennis, 1998). Various options offered for bone graft are autograft, allograft, xenograft, and alloplast. Autograft are tissue transferred from one site to another in the same individual and are considered the most suitable method of bone grafting, the allograft is a transplant material from different genetics but belonging to the same species, whereas xenograft is a graft material from a donor of another species, and alloplasts are graft materials extracted from inorganic, synthetic or dead species (Stephen T, et all, 2009). According to Stuart, Weinberg and Tarnow, although the use of autogenous bone graft is now well accepted but also has disadvantages such as the limitations of donors, the requirements for obtaining graft materials, and also the relatively expensive price (Stuart et al 1998)

Over the last 30 years, various variations of synthetic bone graft have been widely studied in order to minimize the risk of disease transmission. The advantages of synthesis graft include material availability, sterility, and low morbidity (Kartono et al, 2014). Humans and animals have the same complex dental structure, which consists of three layers, namely enamel containing 90-96% hidroksiapatit material, 1% -2% organic material and 3% -4% H2O, while the second layer is composed of a tooth crown dentin 70% hydroxyapatite, 18% organic and 12% H2O, and the third layer is the outer surface consisting of hydroxyapatite cement (Elkayar et al, 2009). The selection of bovine's teeth as a synthesis graft material is due to bovine's teeth containing many hydroxyapatite elements (Afdal et al., 2016). Hydroxypatite is a crystalline molecule composed of phosphorus and crystalline with Ca10(PO4)6(OH)2 molecular formula are included in the phosphate compound. Because of these components, hydroxypatite is one of the most commonly used materials in biomedical applications because it can repair hard organ by means of bioactivity in rebuilding bone tissue and soft tissue that has been damaged (Kusrini & Sontang, 2012).

In addition hydroxypatite has also been widely applied as a catalyst and adsorbent, as it's porous, inert, durable constituent structure and can serve as a cation exchanger (Wahl & Czernuszka, 2006). On the other hand the needs of beef consumption in Indonesia continues to increase. This result can certainly cause problems for the environment due to untapped cattle waste (Sunarso, Sutrisno, & Sumarsono, 2010). Much of the current research has used bovine's bone for hydroxyapatite material and used as a bone graft material, so the researcher aims to find other alternative method using bovine's teeth. The processing of bovine's teeth into bone-grafted materials can be promising because of the abundant availability of materials, the hydroxyapatite of dairy cattle will be economical and help reduce the waste and is also expected to reduce the dependence of imported bone graft (Afdal et al 2016). But to see the success of synthetic graft from bovine's teeth, the substitute must be biocompatible in order to avoid

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rejection by the host, have no toxic effect or cause injury to biological function (Kartono et al, 2014).

Currently, there is no recent empirical data on the viability of bovine tooth graft on fibroblast cell culture. Therefore, this study aims to present the latest data on the viability of bovine tooth graft on fibroblast cell culture with MTT assay method which is expected to increase the information and knowledge especially in the development of the field of dentistry.

In this study toxicity test will be conducted to determine the bioviability of bovine tooth graft before used in the human body. The bioviability test used is the MTT assay test on bone marrow stem cell culture.

Stem cells are primal cells common to all multicellular organisms that retain the ability torenew themselves through cell division and canbe differentiated into a wide range of specialized cell types. TheMesenchymal and TissueStem Cell Committee of the ISCT proposes a set of standards to define human MSC for both laboratory-based scientific investigations and for pre-clinical studies. These dentifying criteria should not be confused with release specifications for clinical studies, as the current proposal isintended solely as identifying criteria for research purposes. The aim of this position statement is to provide the scientific community with a standard set of criteria, basedon the best currently available data, to define the identityof MSC, recognizing that future research will probablymandate a revision of the criteria as new data emerge. We propose three criteria to define MSC:adherence to plastic, specific surface antigen (Ag) expression, multipotent differentiation potential. First, MSC must be plastic-adherent when maintained instandard culture conditions using tissue culture flasks.Second, ≥95% of the MSC population must expressCD105, CD73 and CD90, as measured by flow cytometry.Additionally, these cells must lack expression (≤/2%positive) of CD45, CD34, CD14 or CD11b, CD79a orCD19 and HLA class II. Third, the cells must be able to differentiate to osteoblasts, adipocytes and chondroblastsunder standard in vitro differentiating conditions (Dominici, et al 2006)

MATERIALS AND METHODS

1. Isolation of Mesenchymal stem cells from Rat Bone Marrow

Five male wistar rats were sacrificed by ether inhalation and femurs and tibia were carefully cleaned from skin by pulling toward the foot which is cut at the ankle bone. Disarticulation of the hip and ankle joint was done, and the extremities of each rat were marked and put in RPMI transport medium. Tibial and femoral bones were separated aseptically at the knee joint in biosafety cabinet. Proximal tibial growth plate was cut together with the attached muscles, and the tibia was divided from fibula. All muscles and connective tissues in the femur were detached from the bone and the femoral condyle was cut. Single-cell suspensions were cultured and expanded with alpha modification of minimum essential medium eagle (α-MEM) (Gibco BRL. Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS)(Gibco BRL) and 1% penicillin/streptomycin, in 25 cm2 tissue culture flask and incubation at 370C in 5% CO2 atmospere. The culture medium was changed twice weekly. When the confluence reached 80%, the cells splitting was done using trypsin. (Lotfy et al; 2014).

2. The BMSCs Phenotypic Characterization with Immunocytochemistry

The cells were blocked by bovine serum albumin, incubated with fluorescein isiothiocyanate (FITC)-labeled monoclonal antibody CD 105 and CD 45 for 60 minutes. Immunostained cells with CD 105 and CD45 were analyzed using fluorescence microscope (Hendrijantini et al, 2015).

3. Bovine Teeth Hidroxyapatitepreparation

The study used incisors and molars freshly extracted bovine teeth.To remove any traces of blood, the teeth were washed in water and then stored in distilled water. This process was repeated three times till it yielded white and clean teeth, and then the teeth samples were dried in the sun for 3 days. The teeth were calcined in the muffle at 735 °C (7 °C/min), and then hold for 1 hr, then left to cool, it was observed that at 450 °C huge amount of vapors evolved from the sample. The calcinations was in humid atmosphere to avoid any dehydration in the furnace, the sample was sintered again to 1150 °C (7 °C/min) for 1 hr. This sintering process took place to ensure that the organics are completely removed and that the material is safe and to avoid any microbial contamination. Small particle powder using bone miller and particle sizes ranged from 355 to 710 μm and stored in sterile packages and sterilization by gamma irradiation (Elkayar, Elshazly & Assaad, 2009).

4. MTT assay

The HA scaffold of samples on MSC cells were determined by the MTT (3(4, 5dimethyl thiazol 2yl) 2, 5 - diphenyltetrazolium bromide) assay was used to assess the cytotoxicity Cells (2×106 /well) were plated in 0.2 ml of medium/well in 96 - well plates. For MTT assay the medium from the wells was removed carefully after incubation. Each well was washed with MEM (w/o) FBS for 2 - 3 times and 200 ul of MTT (5 mg/ml) was added. The plates were incubated for 6 hours in 5% CO2 incubator for cytotoxicity. The suspension was transferred to the cuvette of a spectrophotometer and the OD (optical density) values were read by using Elisa reader at 595 nm(Oliveira, Miziara, Silva, Ferreira, Biulchi, & Alves, 2013)

Cell viability (%) = Mean OD/Control OD × 100%

5. Statistical analysis

The esult are presented as the mean and the standard deviation (SD). ANOVA test was performed to determine the significance of the results between selected groups

RESULTS

Isolation and culture of BMSCs

The culture was observed by using an inverted light microscope. Attachment of spindle-shaped cells to tissue culture plastic flask. After 4 passages were represented a homogenously fibroblastic cell monolayer (Fig 1 and 2)

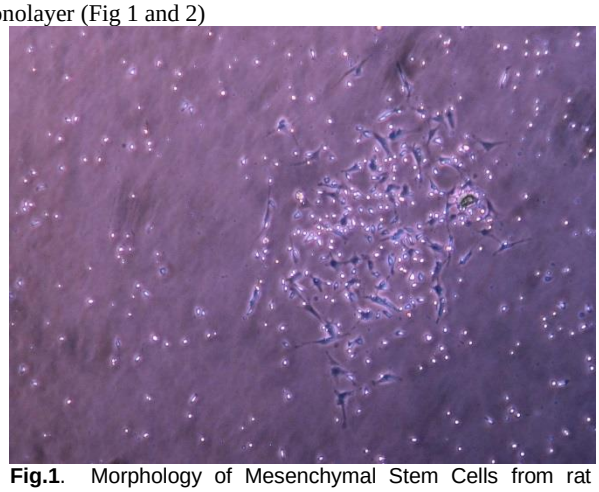

Fig.1. Morphology of Mesenchymal Stem Cells from rat bone marrow, the presence of a homogeneous fibroblast-like population (A) at passage 1 on day 6 of culture (inverted microscope, 200× magnification).

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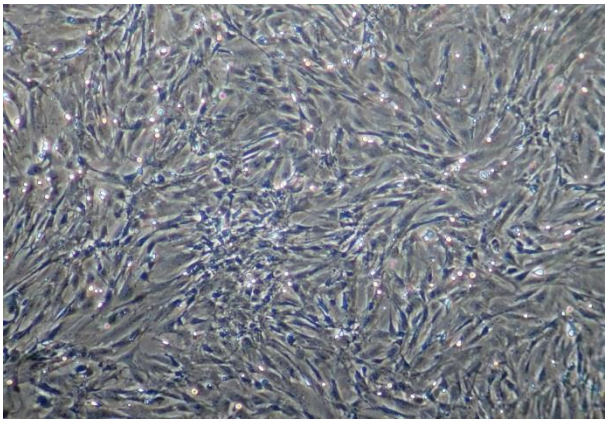

.**Fig.2** Morphology of Mesenchymal Stem Cells from rat bone marrow, the presence of a homogeneous fibroblast-like population (A) at passage 4 on day 15 of culture (inverted microscope, 200× magnification).

Characterization BMSCs with immunocytochemistry. The expression of cell-surface antigens by immunocytochemistry was evaluated. Immunocytochemistry showed that BMSCs expressed strong positive for CD105 and negative for CD 45 at passage 4 (figure 3 and 4).

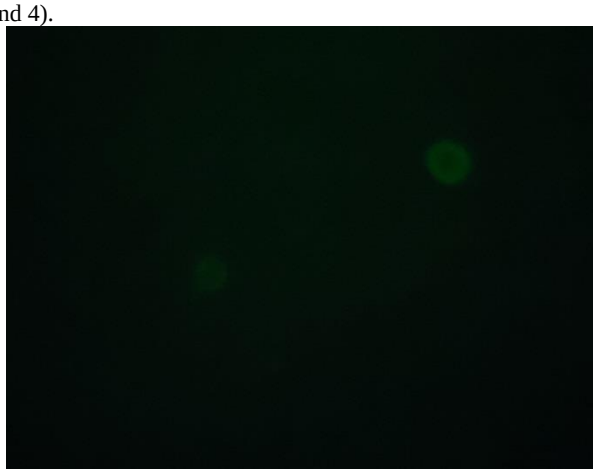

Fig 3 Immunocytochemistry indicated that bone marrow stem cells were negative for cell surface markers negative for CD45 (immunofluorescence microscope, 100× magnification)

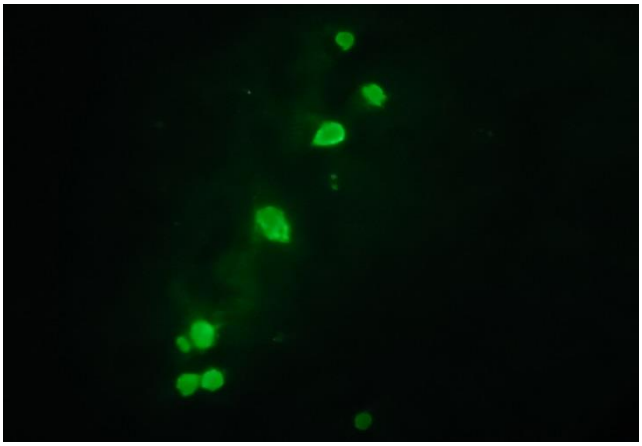

Fig. 4 Immunocytochemistry indicated that bone marrow stem cells were positive for cell surface markers positive CD105 (immunofluorescence microscope, 100× magnification).

Value of cell viability on BTHA scaffold material 10% was 97,1% and 50% was 94,2% and 100 % was 82,7% (Table. 1)

Table.1. Result MTT Scaffold BTHA

No	C M	Ccells	PBS	10%	50%	100%
Amount	0,213	2,931	0,189	2,852	2,772	2,462
Mean	0,043	0,586	0,038	0,570	0,554	0,492
Viabilitas amountcells %				97,1	94,2	82,7

CM = control media
C cells= control cells
PBS = Phospat buffer saline

BMSCs (2×106 cells) were able to proliferate onto BTHAscaffold in standard medium, as by the MTT assay with concentration 10%, 50% and 100% after cell seeding (Fig.5). These results demonstrated that the BTHAscaffold were biocompatible and non-toxic for cell growth

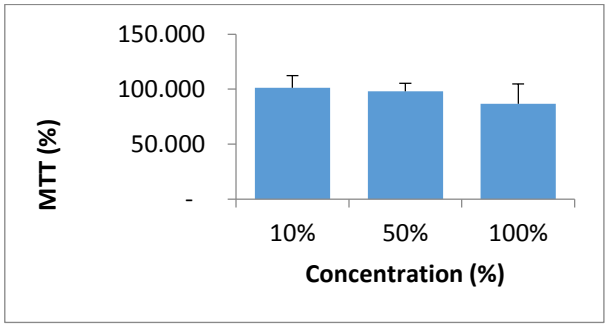

Fig.5 MTT assay results bone marrow stem cells adhesion on different concentration BTHA scaffold. There are significant differences in cell adhesion between concentration BTHA scaffolds (* p≤ 0,05).

DISCUSSION

In general, the bovine's's tooth structure consists of a dental crown, cervical area and the root of the tooth consisting of dentine, enamel, and cementum. As well as the lingual, vestibular (labial or buccal), occlusal, and two contact surfaces (Budras et al., 2011). Animal and human teeth share the same complex structure, consisting of three layers of enamel containing 90-96% hydroxyapatite, 1% -2% organic material and 3% -4% H2O, while the second layer is dental crown consisting of 70% dentine hydroxyapatite, 18% organic and 12% H2O, and the third layer is the outer surface consisting of hydroxyapatite cement (Elkayar, Elshazly, Assaad, 2009). Hydroxyapatite, dentine and cementum are included in type 1 collagen and growth factors such as bone morphogenetic proteins (BMPs). Several studies have isolated BMP from dentine, enamel, and cement of bovine's teeth. (Young-Kyun, et all, 2013)

Hydroxyapatite is a crystalline molecule having a nucleus composed of phosphorus and calcium with the formula Ca10 molecule (PO4)6(OH)2 which is included in calcium phosphate compounds group. As is known bovine's bone and dental structures do not show significant differences. Bovine's bone has been widely used for grafting, tracing, filling or replacing bone, and in the maintenance of dental tissue due to its good biocompatible nature with hard tissue, bioactivity in rebuilding damaged soft tissue and also in soft tissues it self (Kusrini and Sontang, 2006). Hydroxyapatite is used in the medical world because it has properties that can adapt well to damaged bone tissue and also in soft tissue despite its low degradation rate, high osteoinduction properties, non-toxic, non-inflammatory and immunogenic.

The hydroxyapatite crystalline structure can be distinguished by two, i.e manoclinic and hexagonal. In general, the synthesized hydroxyapatite has a hexagonal crystal structure. The

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structure consists of a tetrahedral PO4 arrangement bound by Ca ions. The monoclinic structure can be found if the hydroxyapatite formed is really stoichiometric. The Ca/P ratio of hydroxyapatite is 1.67 and its density is 3.19 g/ml (Ferraz et al., 2004). As known, hydroxyapatite is included in the apatite class (consisting of Ca and phosphate) with the general formula Ca5(PO4) 3OH, and Ca10(PO4)6(OH)2 cell formula. In hydroxyapatite, Ca and phosphate are arranged such that the four Ca atoms are surrounded by nine O atoms of the phosphate portion at M1 position, and the other six Ca atoms are surrounded by the remaining six O atoms. Phosphate part at M2 position. M1 and M2 are crystallographic positions for all Ca atoms. Regardless of its origin, hydroxyapatite contains other elements such as phosphate ions (PO33-), chloride ion (Cl-), flouride ion (F-), and hydroxyl ions (OH). PO33- and Cl- are reported to weaken the hydroxyapatite structure, whereas F- and OH are known to increase apatite strength.

Hydroxyapatite has been widely applied in the biomedical field especially in the regeneration of bone tissue. The utilization of hydroxyapatite is due to non-toxic, biocompatibility, non-inflammatory, non-inflammatory hydroxyapatite, and mesoporous structure of hydroxyapatite. Many researchers have reported an increase in osteogenesis using hydroxyapatite. In addition to increasing osteogenesis, hydroxyapatite can also be used as a coating material. Hydroxyapatite is usually coated on the surface of the implant material thus increasing the bioactivity of the implant. In addition, hydroxyapatite is also suitable as a drug carrier (especially proteins) to targeted cell sections thus helping to stimulate the growth of osteoblastik cell. Hydroxyapatite synthesis shows a strong integration to host hard tissue. Chemical bonding to tissues causes hydroxyapatite as a promising application compared to allograft or autograft

Hydroxyapatite has the ability osteoconduction, and osteoinduction so that can stimulate osteogenesis. The definition of osteoconduction is its function as a scaffold, bone graft becomes a medium for stem cells and osteoblasts to attach, live and thrive in bone defects. Osteoconductive graft can stimulate bone growth and cause bone aposition of the existing bone. Osteoconductive trait of a material are influenced by its shape and structure, such as a degree of porosity, porous size, inter-porous relationships, and surface roughness. Hydroxyapatite is osteoconductive,, which is able to induce and stimulate stem cells and osteoblasts to proliferate and differentiate in the formation of new bone or bone regeneration process. The process of osteoinduction works to stimulate osteogenesis, meaning that bone graft actively stimulates and induces stem cells and osteoblasts from adjacent tissues to proliferate and differentiate in new bone formation. Some growth factors that play a role in the differentiation and proliferation of osteoblasts include bone morphogenic proteins (BMPs), platelet-derived growth factors, insulin like growth factors (I and II), fibroblast growth factor, epidermal growth factor, TGF-β (β1 and β2) and retinoic acid.

In addition to bone regeneration, several studies have also shown that hydroxyapatite can help regenerate periodontal tissue. One was in a study that added hydroxyapatite as a material to coat the implant surface. The study found osteoblastic cells on the hydroxyapatite surface initiating part of the mineralized osteoid formation. The fully formed osteoid then becomes fully mineralized bone and produces a strong bone bond with the hydroxyapatite surface. By 6 months implantation and observed in periodontal defects, there is a small apatite crystal in the middle of the aggregate between the relatively large synthetic hydroxyapatite crystals. Clinical and radiological parameters such as probing depth (PD), clinical attachment level (CAL), intrabony defect depth and percentage of disability are commonly used to evaluate periodontal regeneration. Within 9 months showed that regenerative effects on the superiority were observed with hydroxyapatite compared to OFD groups. Although it is a widely used type of material in the clinic, inconsistent cell reactions (depending on surface properties) lead to restrictions on its use in the clinic. Several hydroxyapatite with modification are also shown to improve protein adsorption (Shue, Yufeng, Mony, 2012).

Mizuki Suto et al., Showed that nano-hydroxyapatite was able to induce the phosphorylation of p38 mitogen-protein (MAP) kinases and this phosphorylation increased BMP-2 expression of gene and protein levels that govern bone formation (Shui et al, 2013). In the Kawai and Urist 1989 studies, it has been shown that bovine's teeth contain comparable osteoinduction proteins in the bone morphogenetic range of bovine bone protein (Kawai & Urist, 1989).

This study using hydroxyapatitie Bovine tooth graft which is a synthesis bone graft type that is processed from bovine's teeth has a 97.1% bioavailibility against bone marrow culture stem cell. This is in accordance with K. Moharamzadeh et al research using dental bovine's dentine as bone graft (in vitro) said; besides being biocompatible, dental bovine's dentine also stimulates dentine proliferation, new bone formation by osteoblasts, fibroblasts and increases cell viability. In addition, in vivo study of K Moharamzadeh et al., on implantation test also confirmed the results of in vitro test, dentin dental bovine's is biocompatible and does not cause inflammatory reaction (Moharamzadeh et al, 2008). In addition to containing hydroxyapatite, Kawai and Urist 1989 research also found that bovine's teeth contain bone morphogenetic protein (Kawai & Urist, 1989). Due to the nature and content of bovine's teeth, the bovine's teeth are good for graft use.

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