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Mesenchymal Stem Cell with Chitosan-Carbonate Apatite Scaffold (In
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Osteogenic Potential Differentiation of Human Amnion Mesenchymal Stem Cell with Chitosan-Carbonate Apatite Scaffold (In Vitro Study)

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Background: Tissue engineering based approaches have received much attention. Incorporation of chitosan and carbonate apatite (CA) improve its capability. Human mesenchymal stem cells (hMSCs) is viable for xenogenic transplantation. The purpose of this study was to fabricate and evaluate the osteogenic potential differentiation of human amnion mesenchymal stem cell with carbonate apatite–chitosan scaffolds (CA-ChSs) for tissue engineering. **Method:** Human amniotic membrane was procured from using cesarean section. Soncini's protocol was employed for the isolation procedure. The cells cultured on collagen-coated dishes using Dulbecco's minimal essential medium (DMEM)/F12 (1:1). A chitosan powder of medium molecular weight deacetylated chitin, poly(D(glucosamine) was used and mixed with CA. Immunocytochemistry and flowcytometry used for phenotypic characterization of hAMSC. **Result:** Amniotic membrane obtained using cesarean section under aseptic condition did not exhibit any growth of cell cultures which were not contaminated. Immunocytochemistry testing revealed that the target cells expressed strong mesenchymal stem cell marker CD 105. Characterization at passage 10 showed that CD44 was the most significant and abundant surface receptors. The number of viable cells in chitosan-carbonate apatite was 66.59%. Scanning electron microscope (SEM) observation revealed that CA-ChSs had three-dimensional structure with many pores and hAMSc could attached and proliferation among the porosity of the scaffold. The formation of calcium in the cell as an indicator of osteoblast cells was detected using Alizarin Red solution. **Conclusion:** hAMSc harvested from human amniotic membrane seeding in CA-ChSs had the capability for in vitro osteogenesis makes them be the one of the potential options for bone tissue engineering.

Keywords: human amniotic membrane mesenchymal stem cells, chitosan-carbonate apatite, scaffold, MSC phenotypic characterization, MTT assay.

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INTRODUCTION

Bone tissue regeneration using the tissue engineering technique has been attempted in order to solve problems, such as complications in wound healing, donor pain, insufficient supply of bone,

immunogenic reaction and disease transmission from donor tissues and fluids. Many efforts have been conducted to create three dimensional scaffolds that provide adequate support as extracellular matrix that allows the cells to proliferate, differentiate, and maintain bodily functions. The property for an ideal scaffolds are non-toxic, suitable microstructure, controllable biodegradability and, as well as suitable mechanical properties. Scaffolds are expected to be capable of cell adhesion promotion and retaining their functions.^{1,2,3,4}

Chitosan can be generated by the deacetylation of chitin and is composed of glucosamine with a variable frequency of N-acetyl-D-glucosamine units.^{4,5} The mechanical strength of chitosan can be varied by altering its microstructure, molecular weight, degree of deacetylation, and crystallinity. Recent studies have shown chitosan promotes osteoblast growth and differentiation in cell culture and shown favorable properties such as bacteriostatic, hemostatic, and cholesterol lowering properties.^{6,7} However, there still exist some problems, such as allergic reactions and low solubility, because of the low degree of deacetylation of chitosan. Recently, highly deacetylated chitosan has been developed and is expected to solve these inherent problems of presently available chitosan.⁸

It is difficult to conclude that a single scaffold using an organic material possesses all the criteria required for successful bone tissue engineering. Chitosan itself has numerous advantages in biomedical applications, but is not osteoconductive and has not enough bone formation ability. One approach to overcome these disadvantages is to design composite substances that combine the strengths of different materials and minimize their drawbacks. Incorporation of chitosan and calcium phosphate substances for example hydroxyapatite (HA), β -tricalcium phosphate (β -TCP), and carbonate apatite (CA) could improve mechanical properties and enhance biological properties as well.^{9,10} Carbonate apatite-chitosan scaffolds (CA-ChSs) had a three-dimensional interconnected porous structure, good retentive form, and the ability to support the proliferation and differentiation of osteoblasts.¹¹

Human mesenchymal stem cells (hMSCs) is viable for xenogenic transplantation because of its favorable properties such as low immunogenicity, immense proliferation rate, and the potential to multi-differentiate. hMSCs serves as the best option for seeding source for tissue engineering. Mesenchymal stem cells (MSCs) derived from bone marrow is currently the best source of autologous stem cells, and often used as seeding cells for cell therapy and tissue engineering.^{12,13} MSCs best properties are its potent regeneration potential and immunosuppressive properties which proves important for the use of allografts. The use of MSCs have already been applied for clinical trials in various studies, such as hematopoietic facilitation and immune reconstitution after hematopoietic stem cells transplantation.^{14,15}

The human placenta especially the amniotic membrane is source of vast amounts of stem cells which is comprised of epithelial and stromal cell. There are recent reports that have shown stromal cells of the human amniotic membrane, also known as human amniotic mesenchymal stem cells (hAMSC), has similar characteristics to bone

marrow mesenchymal stem cell. hAMSC was proven to be have the ability to differentiate itself into three forms of germ layer whilst having anti-inflammatory and low immunogenicity properties.^{14,16} Clinical Application of human amniotic membrane-derived stem cell has advantages over autogenous bone marrow-derived stem cell, this is due to lack of morbidity during procurement procedures and the number of stem cells unlimited available. Moreover, patient's age and health condition highly influence the quality of autogenous BM-MSC. In the case of embryonic stem cells the isolation of hAMSC do not cause any harm to the embryo therefore leaving no any legal and ethical issues.¹⁷

This study is aimed to fabricate and evaluate the osteogenic potential differentiation of human amnion mesenchymal stem cell with carbonate apatite-chitosan scaffolds (CA-ChSs) for tissue engineering.

MATERIALS AND METHODS

Human Amniotic Membrane Procurement

Human amniotic membrane was procured from using cesarean section which was performed at the Central Operating Theatre RSUD Dr. Soetomo Teaching Hospital Surabaya. Committee for Ethics on Health Researches, RSUD Dr. Soetomo legally and ethically approved the methods for this study. Fresh amnion was mechanically extracted from the chorion, washed three times in phosphate buffered saline (PBS) to remove excess blood, and soaked in Ringer Lactate solution containing gentamycin and amphotericin B based on Hendrijantini.¹⁸

Isolation and Culture of hAMSC

Isolation and culture of hAMSC were performed at Stem Cell Research and Development Center, Airlangga University, Surabaya. Soncini's protocol was employed for the isolation procedure. The amniotic membrane was cut into small fine pieces and subjected to 0.25% trypsin to remove the epithelial cells, and subsequently after centrifugation the supernatant was removed in which this protocol was done twice. Crushed tissue was then subjected to PBS containing 0.75 mg/ml collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and 0.075 mg/mL DNase I (Takara Bio, Shiga, Japan), incubated at 37°C for 60 minutes. The sample was then filtrated using cell strainer and pellet collection after centrifugation for 5 minutes the cells were finally obtained. The single cells collected then were cultured on collagen-coated dishes using Dulbecco's minimal essential medium (DMEM)/F12 (1:1) (Gibco BRL, Gaithersburg, MD, USA), added with human leukemia inhibitory factor (10 ng/mL) and fetal bovine serum (Gibco BRL). Then every three days' medium was changed and when confluence reached 80% cell splitting was done employing trypsin. Half to two thirds of the

cells were then replated using the same medium onto new dish.^{19,20}

Preparations of CA-ChSs

A chitosan powder of medium molecular weight deacetylated chitin, poly(D(glucosamine) from Sigma-Aldrich was used throughout the experiments. 200 mg of chitosan powders were dissolved in 5 ml acetic acid in room temperature, shaken for 15 min, neutralized using 15 ml NaOH solution in order to obtain chitosan gels. Then 100 mg of CA were homogenously mixed with the chitosan gels and centrifuged at 1500 rpm for 10 min to prepare CA-Ch gels. After the removal of excess water, CA-Ch gels was then transferred into cylindrical molds (diameter: 5 mm, height: 2 mm), frozen at -80°C for 2 hours and transferred into the freeze-drying machine.¹¹

In order to remove alkali salts as ions, desalination of CA-ChSs was conducted. One hundred microliters of distilled water were added to non-desalinated CA-ChSs and transferred into a dialysis tube. The tube was then placed in tap water for 24 hours and frozen at -80°C for 2 hours. Desalinated CA-ChSs was obtained after drying in the freeze drying machine at -54°C for 24 hours (figure 1). All the materials were exposed to UV light for 2 hours of sterilization before using them in cell culture experiments.¹¹



Figure 1.
Chitosan scaffold

Phenotypic Characterization of hAMSC

Immunocytochemistry

Cultured cells were then plated onto coverslips and after having incubated at 37°C for 1-2 hours, preserved with formaldehyde 10% for 15 minutes. The coverslips were then rinsed four times using PBS and dried for few minutes. The cells were blocked using PBS and FBS 1% for 15-30 minutes and then using PBS the coverslips was washed four times. The cells were then applied with FITC-labelled monoclonal antibody anti-human CD 105 and CD 45 and incubated for 60 minutes. The cells were rinsed with PBS twice having done so the cells

were ready for analysis using fluorescence microscope.

Flowcytometry

Cultured hAMSCs were then trypsinized and suspended in DMEM, washed with PBS, and preserved in formaldehyde 10% for 10 minutes and finally immersed in 10% BSA for one hour. The cells were incubated using Human MSCs Analysis Kit (BD StemflowTM, BD Biosciences) alongside primary antibodies mouse antihuman CD44, CD73, CD90, CD105, and negative cocktail containing CD45, CD34, CD19, CD11b, HLA-DR for 40 minutes. Unbound antibodies were then removed by washing using PBS. FITC-conjugated anti-mouse antibody were used to label the bound primary antibodies then washed with PBS. Subsequently the cells were analyzed using FACS Calibur flowcytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Proliferation Measurement on CA-ChSs

Human Amniotic Mesenchymal Stem Cells were obtained by trypsinization from a petri disc that contains 2×10^6 cells and resuspended in DMEM/F12 that contains 10% FBS, 100 U penicillin, 0,1 mg/mL streptomycin and 2 mM L-glutamine and centrifuged. Cells were added into culture well 96 (M96) to a density of 5×10^4 cell/well and incubated for 24 hours in 37°C and CO_2 5%. After getting 80% of cell proliferation, CA-Ch scaffold was put into the well and 100 μL of medium was added to each well. The cells were incubated again for 20 hours in 37°C and CO_2 5%. 5 mg/mL of MTT reagent (25 μL /well) was added to each well, incubated for 4 hours and was examined under inverted microscope. Scaffold and medium were removed and sDMSO was added to each well (200 μL /well). The absorbance was read by using Elisa reader at 595 nm and the number of living cells were determined utilizing MTT assay using Cell Counting Kit.

SEM Images of CA-ChSs.

Scaffold that has been seeded with hAMSC, fixated with glutaraldehyde 2% for 2-3 hours at 40°C , washing by PBS solution 3 times for each 5 minutes. After that the solution was changed by osmic acid 1% for 1-2 hours and continued washing with PBS solution like before. Dehydration with multiple concentrated alcohol like 30%, 50%, 70%, 80%, 90% and absolute, 15-20 minute for every concentration. The following solution was Amyl acetate absolute as preservative solution until it becomes dry. Scaffold was dried with critical point drying (CPD) device. After sticking to pad stud (holder) with special glue and coating with pure gold with vacuum evaporator, scaffold was ready to examine and photo with scanning electron

microscope (JEOL JSM-T100 Scanning Microscope, Japan).

Culture of hAMSC in Osteogenic Medium

Cultured hAMSCs were detached using $2 \times$ trypsin solution and seeded into 24-well microplate containing osteogenic medium, which composed of α -MEM medium supplemented containing $50 \mu\text{M}$ ascorbate phosphate, $10 \mu\text{M}$ glycerol phosphate and $0.1 \mu\text{M}$ dexamethasone, for osteogenic study. The medium was changed every 3 days. The cells were cultured in the osteogenic medium for 21 days to induce osteoblastic differentiation. After complete differentiation had been established morphologically, the cells were used for histochemical staining and immunohistochemistry studies. Cell fixation was performed by first detaching the cells from the petri dish of both media, followed by seeding into 24-well plate containing 10% formaldehyde. The presence of calcium deposits in the cultures was determined using Alizarin Red stain according to a standard protocol to evaluate osteogenesis.

RESULT

Human Amniotic Membrane Procurement

Amniotic membrane obtained using cesarean section under aseptic condition did not exhibit any growth of cell cultures which were not contaminated. After delivery through cesarian section and the umbilicard cord cutted off, the placenta with the remaining umbilicard cord was then evacuated and placed in a sterile kidney-shape stainless steel receptacle. Then amniotic membrane was peeled off from the underlying tissue which was the chorion and cut out from the placenta. Subsequently the membrane was washed by immersing it repeatedly in two bottles that contains PBS to remove blood and finally immersed in the third bottle containing medium DMEM/F12 complemented with antibiotic and antifungal agents.

Isolation and Culture of hAMSC

Cells growth were observed for the first 24 hours, during that period the cells were mostly round or oval in shape (Figure 2a). After 24 hours observation, most of the cells were adherent to the plate and became spindle in shape. After the third day of observation the cells had grown to a confluence of 90% and congregated to form a swirling pattern (Figure 2b). Cell splitting procedure was performed fourteen times during this study before the cells were ready for subsequent examination.

Phenotypic Characterization of hAMSCs

Immunocytochemistry

Immunocytochemistry testing revealed that the target cells expressed strong mesenchymal stem cell marker CD 105 (Figure 3).

Flowcytometry

In flow cytometry, we did characterization at passage 10. The result showed that CD44 was the most significant and abundant surface receptors. Other MSCs markers showed smaller number of hAMSCs subpopulation, such as CD90 and CD 45 (Figure 4).

Cell Proliferation Measurement on CA-ChSs

The result of toxicity tests with MTT Assay showed that the scaffold Chitosan-Carbonate Apatite (Ch-CA) was not toxic to hAMSC culture. In fact, the number of viable cells in chitosan-carbonate apatite was 66.59%. This suggests that combining chitosan and CA have a good biocompatibility against human amniotic mesenchymal stem cells, for cell proliferation more than 50% on a scaffold chitosan-carbonate apatite.

SEM Images of CA-ChSs

The result of examination and photo with SEM showed that cells could attached to the porosity surface of the scaffold CA-Ch (Figure 5).

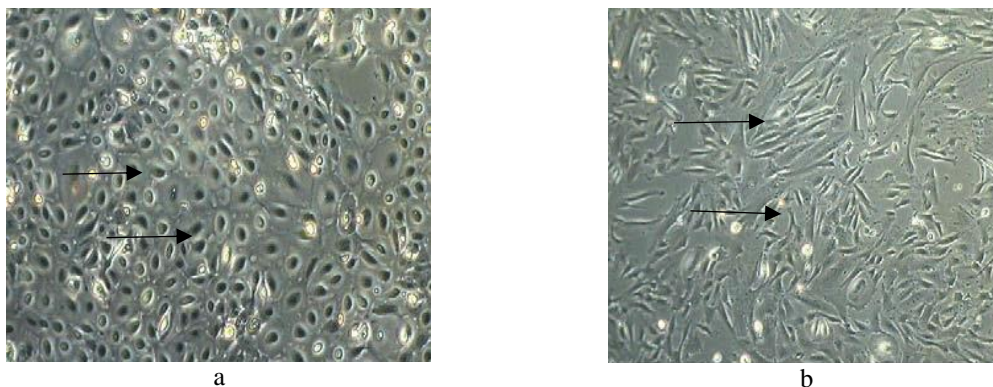


Figure 2.

Culture of hAMSCs. The first 24 hours the cells were mostly round or oval in shape [2] (a) after the third day most of the cells were adherent to the plate, exhibiting spindle-shape or fibroblast-like cell morphology with colonies swirling in pattern, and has reached confluence (b) (inverted microscope, $200\times$ magnification)

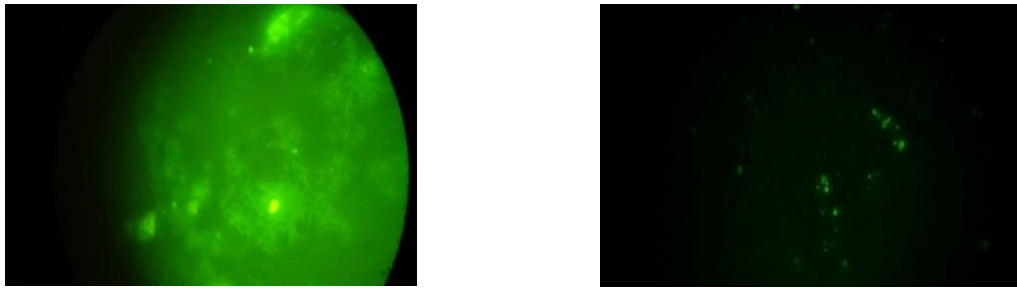


Figure 3.

Phenotypic characterization of hAMSC. The immunocytochemistry result showed that the cells strongly expressed CD105 (*top left*) and weakly expressed CD45 (*top right*)



Figure 4.

The flow cytometry (a) showed that the majority hAMSCs subpopulation expressed CD44 and (b) with smaller subpopulation expressed CD90 and only an even small number expressed CD45

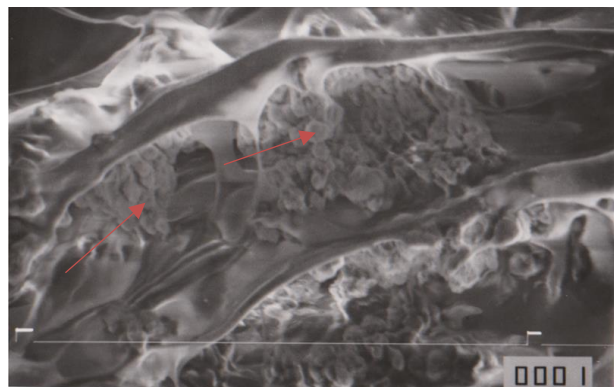


Figure 5.

Cell could attach and proliferate among the pores of the scaffold (SEM,1000x magnification)

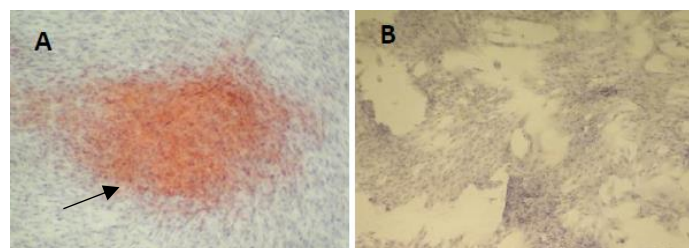


Figure 6.

Staining with Alizarin Red the osteogenic medium exhibited large clusters with bright red appearance indicating the presence of extracellular mineral deposit. (A) while the normal medium showed no color changes detected (B) (Alizarin Red staining, counter-stained with Mayer's hematoxylin, 200× magnification)

Osteogenic Potential Differentiation Study of hAMSC

To test the osteogenic differentiation potential of hAMSC obtained from the process of isolation and culture, conducted in cell culture in a special medium that contains components that can induce osteoblastic differentiation. To determine the degree of osteogenesis hAMSC, the osteogenic culture was added with Alizarin Red solution that could detect the formation of calcium in the cell as an indicator of osteoblast cells (Figure 6).

DISCUSSION

Procurement of amniotic membrane procedure should be executed under highly aseptic condition because slight contamination of the sample, for example in normal delivery, may inevitably cause fungal growing on the cell culture. Isolation protocol was done in the Laboratory of Stem cell Research and Development Center, following Airlangga University protocol and adopted from modified Kamadjaja study.²⁰ Isolated cells showed spindle-shaped fibroblast-like cells which adhered to the petri dish and when cell confluence was achieved the cell colonies formed a swirling pattern. This finding was consistent with the morphological characteristics of mesenchymal stem cell seen in many studies found in literatures.¹⁴ Immunocytochemistry and flowcytometry on the target cells showed insubstantial expression of CD45, the specific phenotypic markers of hematopoietic stem cell.

Results obtained during this research found distinct characterization of hAMSCs. Characterization was done using immunocytochemistry and flowcytometry study on different passage. Immunocytochemistry testing showed that CD105 was strongly expressed with weak expression of CD45 on passage 8. On the contrary, flowcytometry study that was done on passage 10, majority of the hAMSCs subpopulation expressed CD44, smaller subpopulation expressed CD90 and hematopoietic markers CD45. Other studies also mentioned that positive expression of CD73, CD90, and CD105 were found as a characteristics of mesenchymal marker, and other criteria for MSC characterization was only less than 2% of the MSC population will express CD34, CD45, CD11b, CD14, CD79, CD19, and HLA class II.²¹ Similar to this study, Leyva-Leyva et al observed that 82% of the cells population shared CD73 and CD44 antigens, but only 0.08% expressed CD105 on their surface. MSC marker expression can also be significantly influenced by cell passage, CD105 expression reached its maximum (10%) after the first and second passages, but after the third and fourth passages, the expression fell down to 2%. This explain the weak CD105 expression and mostly negative cell subpopulation in 10th passage during flow cytometry examination.²²

In the present study, we attempted to combine chitosan with CA in making CA-ChSs. The optimal amount of chitosan powder was 200 mg and CA was 100 mg.¹¹ Fabrication of CA-ChSs was based on lyophilization technique. Desalination of CA-ChSS was conducted to remove alkaline salt ions. The result was scaffold that had retentive form without brittleness, sponge-like, and not solid. Based on SEM images it was showed that CA-ChSs had three dimensional structure with many pores and hAMSc could attached and proliferation among the porosity of the scaffold. The interconnected porous structure of the scaffolds had good retentive forms and ability to support the proliferation and differentiation of osteoblasts. Scaffolds used for tissue engineering ideally should have porous structure with pore size ranging from 40-300 µm allowing tissue in growth and vascular tissue migration.^{1,4,11}

The result of cell proliferation on CA-ChSs by MTT assay was 66.59%. It means that by combining Chitosan with CA had good biocompatibility to the human amnion mesenchymal stem cells, because cell proliferation more than 50% on CA-ChSs.

Alizarin Red staining was performed to detect osteoblastic differentiation in cell cultures by identifying the presence of extracellular calcium deposits. Stained osteoblasts would appear bright red in color whereas undifferentiated MSCs did not show color changes.²³ This method is highly effective yet simple. In this study, the osteogenic-induced culture showed a significant amount of red-stained cell clusters, indicating that many of the hAMSCs collected were differentiated towards osteoblast lineage in osteogenic environment. The result of this study were consistent with other studies that showed which under certain induction conditions, stem cells isolated from the human amnion membrane could exhibit osteoblast differentiation.^{20,24}

CONCLUSION

Based on the results of this study, it was concluded that hAMSc harvested from human amniotic membrane seeding in CA-ChSs had the capability for in vitro osteogenesis which made them to have the biggest potential for bone tissue engineering. However further studies should be done to explore its utilization potential in bone augmentation reconstruction.

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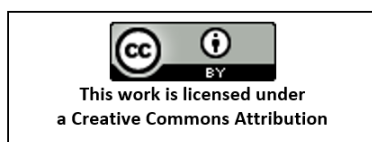
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September 28, 2016

Dear drg Michael J.K. Kamadjaja
Department of Prosthodontic, Faculty of Dental Medicine, Airlangga
University, Surabaya-Indonesia

I am very excited to accept your paper entitled "Osteogenic Potential Differentiation of Human Amnion Mesenchymal Stem Cell with Chitosan-Carbonate Apatite Scaffold (In Vitro Study)". Your paper will be published on the issue of Vol. 5 Number 3, 2016.
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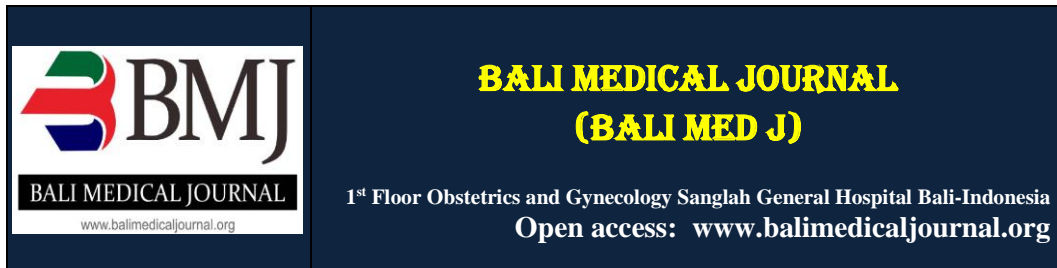
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September 28, 2016

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Department of Prosthodontic, Faculty of Dental Medicine, Airlangga University,
Surabaya-Indonesia

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I am looking forward to work with you and your colleague for further paper.

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