Toxicity and Biocompatibility Profile of 3D Bone Scaffold Developed by Universitas Indonesia: A Preliminary Study

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Abstract. Scaffold as a biomaterial must fulfill some requirem nts to be safely implanted to the human body. Toxicity and biocompatibility test are needed to evaluate scaffold material in mediating cell proliferation and differentiation, secreting extracellular matrix and carrying biomolecular signals for cell communication. An in vitro study with mesenchymal stem cells consisted of direct contact test and indirect contact test using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was conducted on 4 scaffolds made of poly-L-lactic acid (PLA), polyvinyl alcohol (PVA), and hydroxyapatite-poly (vinyl alcohol) composite. There were cells-substrate adhesion impairment, morphological changes, cell death and reduction in cell proliferation seen at 2nd and 6th day in most tested scaffold. Cell count result at day-6 showed proliferation inhibition of more than 50% cell death (inhibition value > 50) in all tested scaffold. In MTT assay, two scaffolds were proven non-toxic. In conclusion, various scaffold materials showed different toxicity effect. The toxicity and biocompatibility profile in this study is a preliminary data for further research aiming to use those local-made scaffolds to fill human bone defect in various needs.

Keywords: Toxicity; biocompatibility; MTT assay; scaffold; mesenchymal stem cell

INTRODUCTION

Tissue engineering relies on biological tissue regeneration which utilizes cells supported by scaffolds and other supporting biomolecules. A diamond concept used as a basic principle in tissue engineering is a collaboration of inductive and conductive substances, scaffolds, and environment which mediate cell growth and proliferation to generate a new healthy tissue. Bone tissue engineering in orthopedic mainly aims to fill bone defect in various pathological condition [1].

Scaffold as a solid three dimensional biomaterial have an analogetic function with extracellular matrix which provides space for vascularization, tissue regeneration and remodeling and also facilitate scaffold-host tissue integration during implantation. A scaffold must be non-toxic and biologically compatible with the host tissue so that cells can safely adhere, proliferate, and differentiate within the scaffold [2]. Scaffold as a biomaterial must fulfill some requirements to be safely implanted to the human body. Toxicity and biocompatibility test are needed to evaluate scaffold material in mediating cell proliferation and differentiation, secreting extracellular matrix and carrying biomolecular signals for cell communication [3, 4].
Due to the variation in mechanical properties required in ‘soft’ versus ‘hard’ issue engineering applications, the fabrication of these two scaffolds generally use different classes of biomaterials. For soft tissue engineering applications, generally a wide variety of polymers are applied. On the other hand, hard tissue replacements, are generally based on combined polymer and ceramic materials, commonly known as biocomposites. Hydroxyapatite (HA) is proven having excellent biocompatibility and osteoconductivity as well as good resemblance with the bony tissues. Poly(vinyl alcohol) is a water-soluble polymer which perform good biocompatibility with 3 dimensional network structure and able to be modified to mimic human tissue. Hydroxyapatite (HA) is proven having excellent biocompatibility and osteoconductivity as well as good resemblance with the bony tissues. Poly(vinyl alcohol) is a water-soluble polymer which perform good biocompatibility with 3 dimensional network structure and able to be modified to mimic human tissue. In addition, good bioresorbability and anti-bacterial properties of Chitosan (CS) makes it a prospective additional component to gain compositional similarity of bone. Ultimately, scaffolds based on pure poly-L-lactic acid (PLA) polymer that has been approved by FDA is also investigated in this study.

The other important aspect of bone tissue engineering is the introduction of bioactive cells into the three-dimensionally porous scaffold either for the sake of biocompatibility testing or introduced along with the scaffold itself to the human body. Mesenchymal stem cells (MSCs) are present in many tissues, easily isolated, easily cultured and rapidly proliferated in the laboratory setting. MSCs serve as a readily available source of undifferentiated cells that are capable to give rise to diverse tissues, including bone, cartilage, adipose tissue, tendon, muscle and other tissues of mesenchymal origin. It does not appear to be rejected by the immune system, allowing for large-scale production, appropriate characterization and testing, a subsequent ready availability of allogeneic tissue repair enhancing cellular therapeutics. All the superiorities of MSCs encourage author to introduce MSCs into the tested scaffold for tissue engineering application. Therefore, in this study, we investigate the biocompatibility and toxicity of the material.

The scaffold toxicity and biocompatibility profile in this study will be used as a preliminary data for further research aiming to use local-made scaffolds to fill bone defect in many bone pathological condition. Scaffold used in this study were originally provided by Universitas Indonesia. This study was conducted as Universitas Indonesia contribution to the field of orthopedic bone engineering and development of nano and advanced technology as stated in Universitas Indonesia strategic plan.

THE METHOD OF THE STUDY

Scaffold materials and fabrication

Scaffolds used in this study were designed and produced by Faculty of Engineering, Universitas Indonesia. Briefly, there are two fabrication technologies used in this study which are rapid prototyping method and freeze drying method. The two methods were selected based on the property of the corresponded material that were investigated.

PLA based scaffold was predesigned by CAD (Autodesk Inventor 2013) software and then rapid-prototyped using our developed Fused Deposition Model (FDM) machine. There were predetermined porosity in the scaffold. The scaffold has a 30% porosity and pore size of around 0.25 mm². In this study, the scaffold coded as Primo.

PVA/Chitosan-HA scaffolds were fabricated using freeze-thaw method. Firstly, PVA was dissolved in distillated water for 7 hours in 90°C, while chitosan was dissolved in acetic acid solution 1.5% v/v for 1 hour. Both solutions were mixed by 80:20 v/v ratio and afterwards being mixed with HA with variation of ratio: 0, 25, 40 and 50% w/v. Prior to freeze-thaw process for 12 hours in each process, the mixture was casted into the molds. Finally, scaffold had been ready for characterization after freeze-drying process.
Biocompatibility in vitro

Cell Culture and Seeding

Mesenchymal stem cells (MSCs) were isolated from human lipid tissue and have been preserved by cryopreservation technique. All procedures have gained ethical approval from University of Indonesia ethical committee. MSCs inside cryovials were taken from liquid nitrogen cryotank and then undergone thawing process in 37°C waterbath. MSCs were put in complete medium consisted of 10% serum, 1% antibiotic (penicillin and streptomycin), 1% antifungal and GlutaMAX™ supplement to improve cell viability and growth and also potentially increasing productivity levels. Cell-medium suspension was centrifuged at 2500 rpm for 10 minutes. The supernatant was recovered and the pellet was re-suspended in 1 ml complete medium. After that, cells were counted by hemocytometer. Cells were seeded into a T25 flask with the density of 5000 cells/cm². Five ml complete medium were added to the flask. Flasks were then incubated in 37°C and 5% CO₂ and observed daily. Every 2-3 days, complete medium changes were done, until mesenchymal stem cells were 90% confluent, and ready for harvesting [4, 8].

Toxicity and Biocompatibility Test

Toxicity and biocompatibility test were conducted using direct contact test and indirect contact test by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. In direct contact test, scaffolds were incubated along with MSCs. Changes in cell morphology, impairment of cell-substrate adhesion, reduction in cell proliferation and correlation between toxic effect of scaffold and cell death can all be evaluated by this test. Those parameters were observed until the 6th day when the cell confluence reach 80% in control well. Harvesting was done in the 6th day by removing scaffolds from the well using sterile tweezer. Medium was completely removed and then wells were cleaned using 500 μl Phosphate Buffer Saline pH 7.4 (Sigma) irrigation. Cells were detached from wells by Tryple Select, followed by counting viable cells using trypan blue [2, 8-10].

In MTT assay, scaffolds and cultured cells are tested with Vybrant® and the absorbance value was determined by ELISA reader at 570 nm wave length. This assay measure cellular metabolic activity via NAD(P)H-dependent cellular oxidoreductase enzymes and may, under defined conditions, reflect the number of viable cells (cell proliferation). Scaffolds were incubated in alpha-MEM (Gibco, USA) at the 1st and 7th day along with MSCs and...
mediums. As much as 10 μl MTT reagent were added to each well and incubated for 4 hours in 37°C. Right after formazan crystals were clearly identified, 100 μl SDS 10% in 0.1 N HCL (stopper) were added. Cells and medium without scaffold was observed as control [3, 8-10].

RESULTS AND DISCUSSION

There were cells-substrate adhesion impairment, morphological changes, cell death and reduction in cell proliferation seen at 2nd and 6th day in most tested scaffold except Primo scaffold (Fig. 2). Those scaffolds showed proliferation activity, however in the 6th day observation, the cell count indicated proliferation inhibition of more than 50% control (Table 1). Primo scaffold showed the best cell proliferation activity among observed scaffolds. We assumed that most of MSCs attached, proliferated and differentiated within the scaffold. To prove this hypothesis, scanning with electron microscope (SEM) is needed in the next study.

FIGURE. 2. Cell morphology and proliferation of various scaffolds were observed at day-6: a). Control; b). PLA (Primo); c). PVA/Chitosan; d). PVA/Chitosan Composite with 40% wt/v HA

All scaffolds in this study were scheduled to have scanning electron microscope (SEM) so we can get a better view of osteogenic activity inside them. Unfortunately, at the time of submission the SEM procedure has not been done yet. That was the reason why we did not do the tryptination phase as an important step of the test. As much as 0.05% Trypsin is supposed to be added to the well to detach cells from scaffold (and later to detach cells from the well) before the morphology and proliferation evaluation under light microscope and before the cell counting step.

Cell count result at 6th day showed proliferation inhibition of more than 50% cell death (inhibition value > 50) in all tested scaffold. We assume that there were trapped cells within scaffolds which couldn’t be evaluated in this study. Moreover, variation of scaffold surface area might affect the test results since the scaffold with wider contact area showed the lesser cell count reflected in the higher percentage of cell proliferation inhibition. This could be a pitfall in this study since the result can be bias.
TABLE 1. Toxicity and biocompatibility of 24 scaffolds and control

<table>
<thead>
<tr>
<th>Scaffolds</th>
<th>Direct Inhibition (%)</th>
<th>Indirect (MTT assay) Inhibition day-1 (%)</th>
<th>Indirect (MTT assay) Inhibition day-7 (%)</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00</td>
<td>100.00</td>
<td>100.00</td>
<td>cells &amp; medium</td>
</tr>
<tr>
<td>PLA (Primo)</td>
<td>54.90</td>
<td>40.25</td>
<td>27.75</td>
<td>PLA</td>
</tr>
<tr>
<td>PVA/Chitosan</td>
<td>94.12</td>
<td>34.00</td>
<td>-45.25</td>
<td>PVA/Chitosan</td>
</tr>
<tr>
<td>PVA/Chitosan with 25% HA</td>
<td>94.12</td>
<td>16.00</td>
<td>-24.25</td>
<td>PVA/Chitosan-HA</td>
</tr>
<tr>
<td>PVA/Chitosan with 40% HA</td>
<td>94.12</td>
<td>30.50</td>
<td>6.50</td>
<td>PVA/Chitosan-HA</td>
</tr>
</tbody>
</table>

In MTT assay, Primo, and PVA/Chitosan with 40% (wt/v) HA were proven non-cytotoxic, while others showed inhibitory activity. Kamal et al. stated that a scaffold is considered as toxic if it inhibits more than 50% of cell proliferation. The least inhibitory value means the scaffold is not toxic. We strongly recommend further study at these scaffolds to get a more complete data as consideration for using them to fill human bone defect.

The data in Figure 3 confirms that the addition of HA enhances the biocompatibility of PVA/CS scaffold. This enhancement is contributed by the chemical and structural similarity to the mineral phase of native bone tissue, which is consistent with the cell morphology and proliferation in Figure 2. Moreover, HA also contribute as a weak alkali inorganic filler, which can buffer acidic from the PVA/CS matrix and further allow the mesenchymal stem cells to live and proliferate. The addition of 40% w/v HA performs the lowest cytotoxicity due to the optimum composition between the organic and inorganic components of the biocomposite.

**CONCLUSION**

Various scaffold materials showed different cytotoxicity effect. Primo as a polymer based scaffold showed the least cytotoxic effect, followed by PVA/CS – 40% HA scaffold. From this study we were able to determine characteristic of each scaffold. Further studies are needed to evaluate toxicity and biocompatibility of these scaffolds in vitro and in vivo before their application to human body as bone defect filler.
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REFERENCES