Cytotoxicity of Gypsum Puger Hydroxyapatite Scaffold on Rat's Bone Marrow Mesenchymal Stem Cells: An In Vitro Study

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Abstract: Hydroxyapatite (HA) is an alloplastic material with molecule formula $Ca_{10}(PO_4)_6(OH)_2$ which is similar with calcium apatite that can be found in teeth and human bones. Hydroxyapatite can be synthesized from natural gypsum as found abudantly in the southern part of Jember district, Puger. Composed with gelatin, hydroxyapatite synthesized from Puger gypsum (HAGP) produce scaffold that might be potential as an alternatif for bone substitute. As a biomaterial, HAGP scaffold must be non toxic to serve a compatible environment for cells. Purpose: To determine the toxicity of HAGP scaffold on rat's bone marrow mesenchymal stem cells. Methods: HAGP scaffold was immersed in cell culture medium with concentration 10mg/mL, 50 mg/mL, 100 mg/mL for 4 days. The filtered immersion medium was then exposed to MSCs cultures. The viability of MSCs were then calculated using MTT method. Results: Cells viability in HAGP 10mg/mL group was 100%, in HAGP 50 mg/mL group was 85,3%, and 78,8% in HAGP 100 mg/mL group. The significance value of 0.016 ($P < 0.05$) indicate that there were significant differences between the groups, but there were no significant difference between HAGP 50 mg/mL group and HAGP 100 mg/mL group . Conclusion: HAGP scaffold indicates as a non toxic material for cells due to high MSCs viability.

1 INTRODUCTION

Hydroxyapatite (HA) with the molecule formula $(Ca_{10}(PO_4)_6(OH)_2)$ is a bioceramic apatite calcium that can be found in human teeth and bones. HA is an alloplastic material produced by reaction of high temperature and crystalline form of calcium phosphate with 1:67 calcium phosphate atomic ratio. In the world of modern medicine HA material is widely used as a substitute for human bones. The properties and advantages of HA are osteoconductive or bone bonds, can grow and develop together with the original bone or good bone regeneration and have a high biocompatibility (Fitriawan, 2014; Mao, 2014)

Hydroxyapatite can be synthesized from a variety of sources such as bones, mussel shells, or gypsum (Fitriawan, 2014; Sedyono and Tontowy, 2008; Balgies, Dewi, and Dahlan, 2011). Gypsum is a mineral mine consisting of various crystals with chemical formula $CaSO_4.2H_2O$ or calcium sulfate dihydrate. The excavation of gypsum material for industrial is abundant in the southern part of Jember, especially in the Puger district (Naini and Rachmawati, 2010). The gypsum product is widely used in building and medicine in the form of hemihydrate powder (Anusavice, Shen and Rawls, 2012). Previous research has successfully synthesized gypsum from puger to hydroxyapatite / gypsum hydroxyapatite Puger (HAGP). Characteristic of HAGP conducted by XRD and

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FTIR test showed similar pattern with Hap 200 japan (standar) (Naini, Ardhiyanto and Yustisia, 2014).

In general, alloplastic material to be used in living organisms must be acceptable by the body. It is necessary to test the biocompatibility of a material to make sure that it has no harmful effects in the biological system (Mahyudin and Hermawan, 2016). Biocompatibility of the material can be measured in vitro by cytotoxicity test with Cultured Mesenchymal Stem Cells (MSCs). MSCs are used because they are highly sensitive to toxic agents (Hendrawan, 2013). The objective of this study was to observe the HAGP scaffold cytotoxicity as an alloplastic material in rat's bone marrow mesenchymal stem cells.

2 MATERIALS AND METHODS

2.1 HAGP scaffold preparation

Solid gelatin was melted using hot water at 60^0C to produced 10% gelatin liquid. 4 grams of HAGP and gelatin liquid were mixed and freeze dried. Scaffolds were then crushed and grinded into 150-355 µm sized-particles.

2.2 Cytotoxicity test

HAGP scaffolds were immersed in cell culture medium at concentration of 10 mg/mL, 50 mg/mL, 100 mg/mL for 4 days. The immersion medium of HAGP was centrifuged 2000 rpm for 3 minutes and the supernatant was filtered. The supernatant was diluted to a concentration of 1000 µg / ml.

MSCs were cultured in 96 well plate at density of 5 x 10^3 cells/well using HAGP immersion cell culture medium and then incubated for 24 hours at 37^0C and 5%CO_2. All groups were made in 3 replications. After 24 hours of incubation, 25µL MTT were added to each well and incubated for 4 hours. The viability of MSCs was measured using ELISA reader at 595 nm wavelength. The value of OD is used to calculate the percentage of viable cells by using the formula:

$$\% \text{ viable cell} = \frac{\text{OD treatment - OD medium}}{\text{OD control cell - OD media.}} \times 100\%.$$

3 RESULTS

The viable rat's MSCs can be shown in Figure 1. HAGP concentration of 10 mg / mL, Figure 2. HAGP concentration of 50 mg / mL, Figure 3. HAGP concentration of 100 mg / mL.

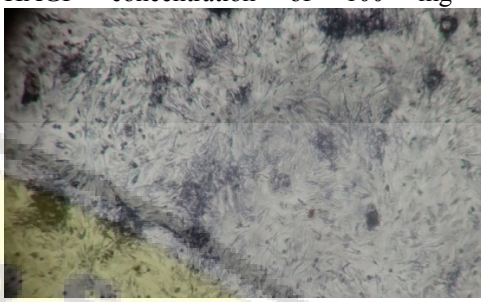

Figure 1: Viable cells after incubated with HAGP immersion medium at concentration of 10 mg / mL.

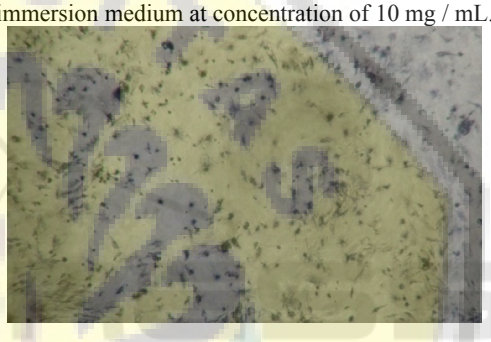

Figure 2: Viable cells after incubated with HAGP immersion medium at concentration of 50 mg/mL.

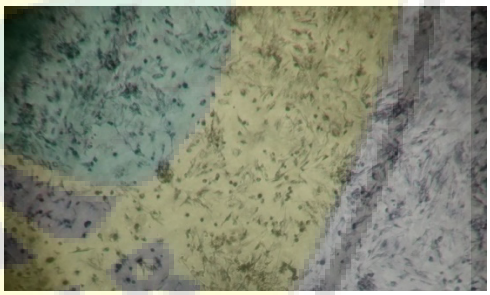

Figure 3: Viable cells after incubated with HAGP immersion medium at concentration of 100 mg/mL.

HAGP Scaffold cytotoxicity was examined using MTT method to obtained viable cells percentage (table 1).

Table 1. Percentage of viable rat's MSCs after incubated with HAGP Scaffold.

No	K.M	K.Cells	10 %	50 %	100 %
1	0,041	0,562	0,620	0,470	0,452

2	0,041	0,620	0,550	0,506	0,454
3	0,043	0,604	0,612	0,504	0,408
4	0,043	0,581	0,552	0,488	0,405
5	0,045	0,564	0,596	0,534	0,578
Total	0,213	2,931	2,930	2,502	2,217
Mean	0.043	0,586	0,586	0,500	0,459
Number of % viable cells			100	85,3	78,8

In the HAGP 10% group showed highest percentage of viable cells (100%) compared to HAGP 50% (85,3%) and HAGP 100% (78,8%). A comparison of viable cells can be seen in the histogram of figure 4.

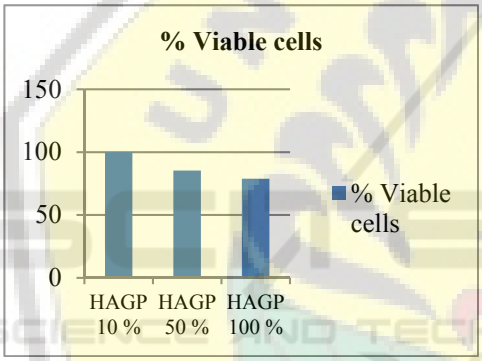

Figure 4. Histogram of viable rat's MSCs percentage after incubated with HAGP immersion medium.

Statistical differences were determined by Kruskal Wallis test because the data distribution is not normal. The results of the analysis can be seen in table 2. Differences were considered statistically significant at $p<0.05$

Table 2. Kruskal Wallis result of viable MSCs after incubated with HAGP immersion medium at concentrations of 10%, 50% and 100%.

Scaffold	Concentration			P
	10%	50%	100%	
HAGP	100,25 ± 9,20	84,35 ± 5,59	77,05 ± 15,27	0.016*

Description: * significant $\alpha = 0.05$.

Based on table 2. the significance value 0.016 ($p <0.05$) showed that there was a significant difference between the hydroxyapatite group of Gypsum Puger.

4 DISCUSSION

In this study, puger gypsum can be synthesized into a hydroxyapatite powder with a temperature of 100 ºC for 20 minutes using a microwave. HA powder were then mixed with gelatin liquid up to 10 ml and freeze dried with a system of sublimation to produce HAGP scaffold.

Over the past years, interest in *in vitro* research as an alternative to experimental animals in toxicology research continues to increase. Cytotoxicity is an unwanted or harmful toxic effect caused by biomaterials in cell culture systems in vitro. The cytotoxicity test used cell growth rate (cell count) and metabolic activity (MTT) which has demonstrated cytotoxicity levels of puger gypsum hydroxyapatite (Theiszovaa, Jantovaa, Dragunovab, Grznarovaa and Palouc, 2005).

Based on the results of the MTT assay (figure 4), it is clear that the cell's survival is highly dependent on the concentration of the hydroxyapatite material (Sjerobabin, Colovic, Petrovic, Markovic, Zivkovic and Jokanovic, 2016). As the results obtained in Table 1. The HAGP 10% group has the highest percentage of viable cells (100%) compared to HAGP 50% group which has lower viable cells (85.3%) and the HAGP 100% group as the lowest (78.8%). It showed that the higher HAGP concentration the lower the cell survival.

The percentage of viable rat's MSCs in HAGP 100% group is lower than HAGP 10% group. It might due to higher hydroxyl groups in HAGP 100%. The group can be degraded in soaking medium so that it releases hydroxyl ions (OH-). The hydroxyl ions can damage cell DNA by causing various oxidative lesions. This oxidative lesion will result in genomic damage to cell death (Cooke, Evans, Dizdaroglu and Lunec, 2003; Aguiar, Furtado, Repole, Ribeiro, Mendes, Peloso, Gadelha, Macedo, Franco, Pena, Teixeira, Vieira, Guarneri, Andrade and Machado, 2013; Li, 2015).

HAGP can also release calcium (Ca2 +) ions and phosphate ions (PO43-) which are their constituent structures. The greater the concentration of HAGP the higher the Ca2 + and PO43- levels. Excessive levels of Ca2 + can cause cell plasma membranes to become ruptures. The elevation of Ca2 + can

activate transition permeability pore opening composed of proteins so that water and solutes in the cytosol will enter and cause mitochondrial swelling to the occurrence of membrane rupture (Zhivotovsky and Orrenius, 2011).

Excessive calcium ions activated by catabolic enzymes and free radicals can lead to cellular toxicity by damaging cell signaling and mitochondrial functions (Yu, Canzoniero and Choi, 2001). In addition, Ca2 + is able to influence PO43- to induce apoptosis (Adams, Mansfield, Perlot and Shapiro, 2001). Essentially, phosphate ions are essential for the nutrient formation Nucleic and cell membranes. The PO43-balance is needed to generate energy in cell metabolism. However, if PO43- is not in balance, interference may occur with cell signaling and ending in cell death (Osuka and Razzaque, 2012).

Based on the discussion that the percentage of viable cells is higher when the concentration of HAGP is lower. The mean percentage of viable cells was 100% in the HAGP 10% group, while the percentage of viable cells 85.3% in the HAGP 50% group, and 78.8% in the HAGP 100% group. This result indicated that HAGP concentrations of 10%, HAGP 50% and HAGP 100% were not cytotoxic, and HAGP 10% is the safest concentration for bone substitute material because the average survival rate was 100%.

5 CONCLUSIONS

The feasibility of Puger Gypsum Hydroxyapatite scaffold as candidate of bone substitute material was assessed. In the cytotoxicity test indicated that administration HAGP 10%, HAGP 50% and HAGP 100% are not toxic to rat's Meshenchymal Stem Cells, with the highest viable cells reaching 100% in HGP 10% group. The result suggest the potential of HAGP scaffold as an alloplastic material for bone substitution because they support cell viability.

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