Effect of Yellowfin Tuna Bone Derived Hydroxyapatite on Hela and Vero Cell Line Proliferation

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Abstract

This study aimed to evaluate the proliferation of Vero and Hela cells after exposed to yellowfin tuna bone derived hydroxyapatite (THA). Vero and Hela cells were cultured in M199 medium and RPMI respectively, supplemented with 10% FBS, 2% Penicillin and Streptomycin, and 0.5% fungizone. The cells were exposed to various concentrations (12.5 to 100 µg/ml) of THA for 24 and 48 hours. The proliferation of the cells was examined using MTT assay. The results showed that the proliferation percentage of Vero and Hela cells was more than 100% in all treatment groups. ANOVA showed no statistically difference in the optical density (OD) values among the groups of Vero cells exposed to THA for 24 and 48 hours ($p>0.05$). However, OD values of the Vero cells exposed to 25, 50 and 100 µg/ml THA after 48 hours exposure were significantly higher compared to the control group ($p<0.05$). ANOVA showed statistically difference in the OD values among the Hela cells ($p<0.05$) after 24 and 48 hours exposure to various concentration of THA. In conclusion, Vero and Hela cells enhance their proliferation after exposed to THA, suggesting the potency of THA to be used as a biomaterial.

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Introduction

Hydroxyapatite (HA) $Ca_{10}(PO_4)_6(OH)_2$ is a bioactive material that chemically similar to mineral components of normal bone and hard tissues in mammals. This material is one of few materials that supports bone in growth and osseointegration.[1] Nowadays, HA has been extensively applied for medical and dental treatment due to its excellent bioactive, biocompatible, biodegradable, and osteoconductive properties. HA grafts are commonly used for reconstruction of the bone lost during tumor removal or trauma, as well as augmentation for dental implants.[2] A previous study by Okamoto et al.[3] showed that HA may be used as a pulp capping agent since it caused less pulp irritation. An *in vivo* study by Setiawatie et al[4] demonstrated that carbonate HA combined with hyaluronic acid accelerated new bone formation. Another study by Putra et al.[5] showed that HA-gelatin composite based injectable bone substitute had potential to be used as a bone filler.

Naturally derived HA has been used in healthcare from a long time period, but ensuing developments have enhanced their utility in healthcare. Natural HA retains architectural microstructure of bone after deproteinization and is better resorbed by body compared to synthetic HA.[6,7]

Yellowfin tuna (*Thunnus albacares*) is one of the most widely consumed fish in worldwide. People just take the meat of the fish and leave the rest as a waste product. To date, tuna fish waste products, such as tuna bone, only utilized as an additive or filler of fish feed. It was proved that the bone of tuna fish contains high calcium and phosphor.[8] In order to increase its value, considering its high calcium content, tuna bone may be used as a biomaterial source. To possess a clinical value, a biomaterial must be able to contact with tissues of the human body without causing a negative effect to the body. It is not only associated to toxicity, but to all the adverse effects of a material in a biological system as well.[9]

Vero and Hela cells are common continuous cell lines used in research. Vero cells

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are derived from the kidney of an African green monkey (*Cercopithecus aethiops*), whereas HeLa originates from a human cervical cancer tumor. Both cell lines have been used extensively in many biological research studies, including the assessment of the effects of substances on cells at the molecular level. Both cells are suitable model for cytotoxicity *in vitro*.[10,11]

In this study, the effect of yellowfin tuna bone derived HA (THA) on cell proliferation was assessed on Vero cells, as well as on Hela cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Materials and methods

THA preparation

THA was obtained from bone tissue of tuna bone. The bone was heated with pressure (80 kPa) for 1.5 hour, then dried with oven at constant temperature for 8 hours per day for 4 consecutive days. After the heat treatment, the material was grinded into fine powder. The powder was then sintered at 700°C for 5 hours. Finally, the THA powder was sieved at 200 mesh of pore size (74 µm).

Cell Cultures

Vero and Hela cell lines were obtained from Integrated Research and Testing Laboratory Universitas Gadjah Mada. Vero cells were cultured in M199 medium supplemented with 10% fetal bovine serum (FBS), 2% penicillin and streptomycin, and 0.5% fungizone. Hela cells were cultured in RPMI medium 1640 supplemented with 10% FBS, 2% penicillin and streptomycin, and 0.5% fungizone. Both cells were subcultured for the study after 80% confluence was reached.

MTT Assay

The proliferation of the cells was measured by direct exposure of the cells to THA. The suspension of THA was prepared by first mixing the particles in the medium to give a stock suspension with a concentration of 10 mg/mL, then diluting the stock suspension, as necessary, to give suspensions with particle concentrations of 12.5–100 µg/mL. The stock suspension was prepared freshly before each assay.

The cells were seeded in 96-well plates at 10,000 cells/well, incubated at 37°C in a fully humidified atmosphere at 5% CO_2 in air for 24 hours. The medium in the control groups was then replaced by fresh medium, whereas the medium in the trial groups was replaced either by M199 medium or RPMI medium 1640 containing different concentrations of THA. The cells cultured without THA particles were treated as a control group.

Cell proliferation was measured using the MTT (3-(4, 5-dimethyl-2-thya-zolyl)-2, 5-diphenyl-2H-tetrazolium-bromide assay after 24 and 48 hours. The medium was removed and 0.1 mL MTT solution at concentration 0.5µg/µL was added to each well. After incubation for 4 hours at 37°C in a fully humidified atmosphere of 5% CO2 in air, 0.1 mL of 10% sodium dodecyl sulphate (SDS) in 0.1 N HCl was added as a solvent for formazan. Following overnight incubation in the dark, plates were shaken and absorbance was read using ELISA (enzyme-linked immunosorbent assays) reader at a wavelength of 550 nm. Reported values were the means of three replicates and were expressed as percentages of the dye accumulated in relation to the control values.

$$\% \text{ cell proliferation} = \frac{\text{OD treated group}}{\text{OD untreated group}} \times 100\%$$

Statistical analysis

One-way ANOVA followed by LSD was done for statistical analysis with a confidence level of 95% ($p<0.05$) considered statistically significance.

Results

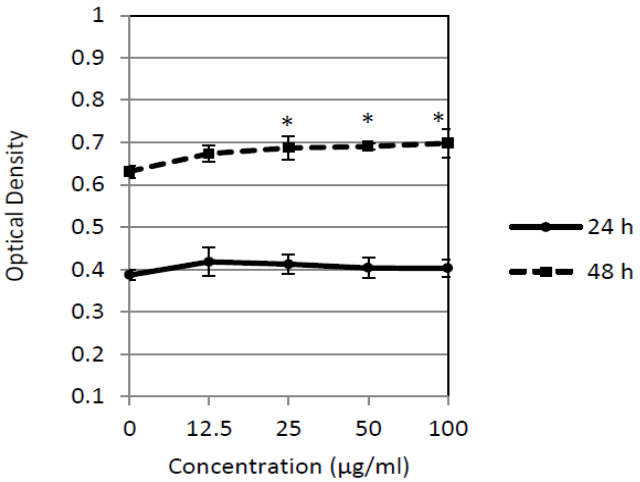

Figure 1. Viability of the Vero cells after treated with different concentrations of THA as determined by MTT assay. Each point represents

the mean+SD of three experiments. Asterisks indicate significant differences (p<0.05) in the OD of THA-treated cultures compared with untreated control.

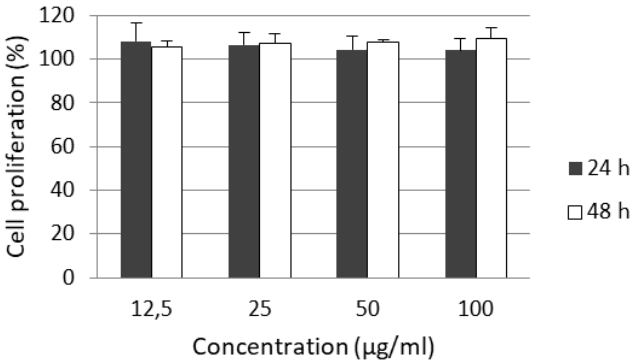

Figure 2. The proliferation of the Vero cells after treated with different concentrations of THA. The data are expressed as a percentage of the untreated cells. Each point represents the mean+SD.

The effect of THA on Vero cells viability measured by MTT test is shown in Fig.1, Fig.2. The results showed that the OD values of all experimental groups was higher compare to the control group. ANOVA revealed no statistically difference in the OD values among the groups after 24 hours (p=0.582) as well as 48 hours (p=0.057) exposure of THA, indicating that Vero cells viability was not affected by THA. However, LSD test showed that after 48 hours exposure, the OD value of the experimental groups (25, 50 and 100 µg/ml) were significantly higher compared to the control group (p<0.05). In addition, experimental data showed an increase to 105.26-109.12% of cell proliferation after the cells were stimulated with THA for 48 hours. The results indicated that the addition of various concentration of THA to the medium for 48 hours increased the proliferation of Vero cells.

Hela cells viability after stimulated with various concentration of THA is presented in Fig.3. The results revealed that the OD value of all experimental groups was higher compare to the control group. ANOVA showed statistically difference in the OD values among the groups after 24 (p=0.000) and 48 hours (p=0.009) exposure of THA. The OD value of the experimental groups (12.5, 25, 50 and 100 µg/ml) were significantly higher compared to the control group (p<0.05). The results indicated that the addition of various concentration of THA to the medium for 24 and 48 hours increased the viability of Hela cells. The percentage of Hela cells proliferation in relation to the control values was 104.35%-108.85% after exposed to THA for 24 hours and 105.28%-109.8% after exposed to THA for 48 hours. These results indicated that THA enhanced the proliferation of the Hela cells (Fig.4).

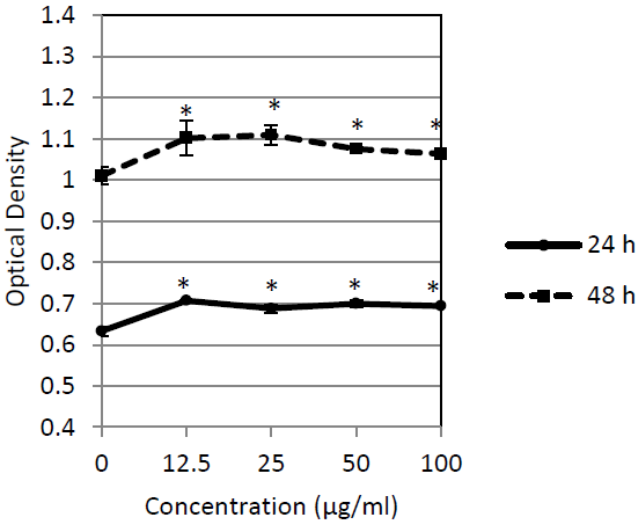

Figure 3. Viability of the Hela cells after treated with different concentrations of THA as determined by MTT assay. Each point represents the mean+SD of three experiments. Each point represents the mean+SD of three experiments. Asterisks indicate significant differences (p<0.05) in the OD of THA-treated cultures compared with untreated control.

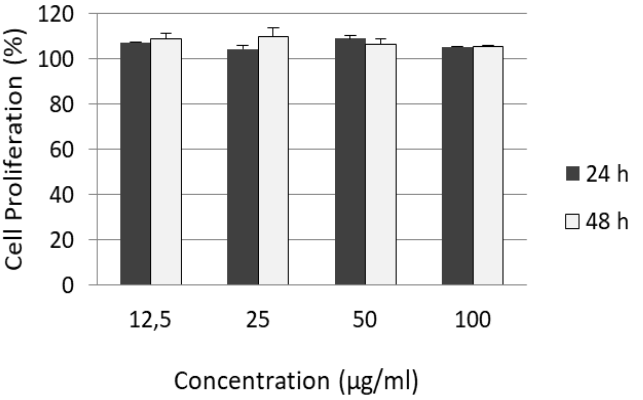

Figure 4. The proliferation of the Hela cells after treated with different concentrations of THA. The data are expressed as a percentage of the untreated cells. Each point represents the mean+SD.

Discussion

The effect of THA on the proliferation of Vero and Hela cells was measured by MTT test. The MTT assay is a quantitative and reliable colorimetric assay that sensitively measures viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are capable to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. This formation production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity. The reaction is mediated by dehydrogenases enzymes associated with the endoplasmatic reticulum and the mitochondria.[12]

The results presented in this study showed that Vero and Hela cells maintained their viability after exposed to the various concentration of THA. Indeed, the number of viable cells after treated with THA was more than untreated control group. The data showed that the percentages of cell proliferation of both cells tested were more than 100%. The percentage of cells proliferation was increased slightly over the time in the treatment groups. Pursuant to ISO 10993-5[13], percentages of cell viability above 80% are considered as non-cytotoxicity, thus these findings proved that THA had no cytotoxic effect on Vero and Hela cells. The results also showed that the material tested promoted Vero and Hela cells proliferation.

HA is a calcium phosphate similar to the human bone and teeth in morphology and composition.[14] An important characteristic of HA is its stability when compared to other calcium phosphates. Thermodynamically, HA is the most stable calcium phosphate compound under physiological conditions as temperature, pH and composition of the body fluids.[15] THA considered as a natural HA which might retained architectural microstructure of bone after deproteinization and might be better resorbed by body compared to synthetic HA.[6,7]

The results of this study proved that THA had a potency as a biomaterial as it promoted the proliferation of the Vero and Hela cells.

Conclusions

This study provides evidence that THA enhances Vero and Hela cells proliferation.

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Declaration of Interest

No potential conflicts of interest were disclosed.

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