Viability Test of Fish Scales Collagen from Oshphronemus Gouramy on Osteoblast Cell Culture

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Abstract

Collagen is the potential material for periodontal tissue engineering. Fish scales can be used as an alternative source of collagen. The aim of this study was to examine the viability effect of collagen extracted from Oshphronemus gouramy scales on osteoblast cell culture. Osteoblast cell culture was made from the calvaria of baby Wistar rat. Collagen from Oshphronemus gouramy scales was extracted using 6% acetic acid. Collagen extract in different concentrations (0.00625 mg/ml, 0.0125 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, and 0.4 mg/ml) was added into osteoblast cell culture and incubated for 24 hours. MTT assay was performed to observe the viability of osteoblast cells. The data were statistically analyzed using ANOVA. The result showed that there were no significant differences in osteoblast cell viability after the administration of collagen extracted from Ohphronemus gouramy scales at various test concentrations ($p>0.05$).

The percentage of viable cells of osteoblast was upper 50%. Oshphronemus gouramy scales can be a good alternative source of collagen because it has high viability against osteoblast cells.

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Introduction

Tissue engineering is new emerging biotechnology that focuses on the synthesis of new 3D biofunctional materials to serve as porous scaffolds for cell attachment. The main properties of biomaterials were biocompatibility, degradability, and structural integrity. Collagen is an abundant natural polymer in all vertebrates, which provides the major mechanical support for cell attachment. Collagen is the essential structural protein in the extracellular matrix in most connective tissues. Collagen is widely recommended for tissue engineering due to its advantageous properties.[1]

The most of collagen was originated from mammals, such as pig and cow. Because of the outbreak of zoonotic infectious diseases, such as Bovine spongiform encephalopathy (BSE), the use of collagen derived from mammals needs to be considered. In addition, some population were allergic to bovine or porcine collagen. Furthermore, in countries having religious restrictions, the application of mammal isolated-products was strictly prohibited. Hence, it was highly desirable and necessary to explore alternative sources of collagen.[2]

Collagen can also be extracted from skin, scales, bone, and fins of freshwater and marine fishes. The previous study demonstrated that fish scales can be an alternative source of collagen.[3,4] Scales from Oshphronemus gouramy can be extracted to obtain collagen. These source are good substitutes for mammalian collagen. Fish collagen is easier for digestion and adsorption than bovine and porcine.[2,5] Therefore, the aim of this study was to examine the viability effect of collagen extracted from Oshphronemus gouramy scales on osteoblast cell culture using MTT assay.

MTT assay was often used as the golden standard of biocompatibility test. MTT assay aimed to measure cytotoxic effects on cells as an indicator of cell proliferation rates.[6,7] Cytotoxicity test, as a part of the evaluation, was required as a standard screening procedure. One of the methods for assessing the cytotoxicity of the material was using reagent 3-(4.5-dimethylthiazole-2-yl)-2.5-diphenyl tetrazolium

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bromide (MTT assay). The MTT test was performed using osteoblast cell culture from the calvaria of Wistar rat.

Materials and methods

Ethical approval

This research was a laboratory experimental study and received ethical clearance from the Bioethics Committee of Faculty of Dental Medicine, Universitas Airlangga, Surabaya, registered 274/HRECC.FODM/X/2018.

Experimental design

The research methodology was true experimental with posttest-only control group design. Samples were divided into eight groups. Seven groups was treated collagen extracted from Oshphronemus gouramy scales at concentration of 0.00625 mg/ml, 0.0125 mg/ml, 0.025 mg/ml, 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.4 mg/ml, and the one group (control) without treated with collagen extract. The experiment was performed in triplicates (n = 3). The data were statistically tested using Shapiro-Wilk to observe normality data and Levene test for homogeneity. Furthermore, the data were statistical analysis using one-way ANOVA with a confidence level of 95%.

Collagen extraction

The extraction of Oshphronemus gouramy scales into collagen was conducted at Politeknik Negeri Malang, according to Prahasanti et al.[8] Oshphronemus gouramy scales were washed in distilled water and dried under the sun. Oshphronemus gouramy scales (80 g) were soaked in 1 M NaOH solution, at 4° C for 24 hours to remove non-collagenous protein. The NaOH solution was replaced every 8 hours. The scales were then washed with distilled water, defatted with 10% isobutanol and subsequently decalcified using 250 ml of a chelating agent (EDTA) for 8 hours, and then immersed in 800 ml of 0.5 M acetic acid and 0.1 g pepsin enzyme. The mixture was agitated using an ultrasonic device (DAWE) at a frequency of 30-40 Hz for 3 hours at 4° C and passed through filter paper. The precipitate was obtained by adding 0.5M NaCl, collected through a process of centrifugation in small tubes at 4.000 rpm for 10 minutes, and finally, washed in distilled water. The resulting precipitate was lyophilized using a freeze dryer (Virtis) to remove all water content at a condensing temperature of -76° C and an ambient temperature of 23.6° C for 12 hours. The resulting collagen extracted from Oshphronemus gouramy scales was sterilized by gamma-ray radiation (Figure 1).

Figure 1. Collagen extracted from Oshphronemus gourami.

Cell culture preparation

Osteoblast cells from calvaria of newborn 2 days Wistar rats were isolated and cultured according to Jonason and O'Keefe.[9] Osteoblast cell was grown in culture medium (DMEM) and incubated in a CO_2 5% incubator at a temperature of 37^0 C. The osteoblast cell condition while in the CO_2 incubator was closely observed. If the cell is 80% confluent, it is washed twice with Phosphate Buffer Saline (PBS) and added with trypsin-EDTA 0.2% in order to release cells from the flask. The density of osteoblast cells was calculated using a hemocytometer.

MTT assay

Osteoblast cells were transferred to a 96-well microplate at a density of 7.5 x 10^3 cells in 100 µL. Collagen extracted from Oshphronemus gouramy scales at varying concentrations was then added. 25 µL MTT reagent was inserted in each microplate, including the control medium and incubated for 4 hours. Wells containing cells only were used as controls. The plates were incubated and maintained at 95% humidity at 37° C and 5% CO2. Cell viability was determined after 24 hours of culture using the standard colorimetric MTT assay to observe viable

cells.[10,11] 240 µL stopper solution DMSO 0.01% was inserted in each microplate after 4 hours of incubation. The 96-well microplate was absorbed by the ELISA reader at a wavelength of 595 nm with the resulting data being used to calculate the viability percentage of osteoblast cells with the formulation below.[8]:

$$\% \text{ viable cells} = \frac{(\text{OD treatment} - \text{OD media})}{} \times 100\%$$

Note: OD = optical density

Statistical Analysis

The obtained data were tested using statistically procedure. Normal distribution test using Shapiro-Wilk test. Homogeneity test using Levene's test. Statistical analysis was performed to compare between groups. The data were statistically analyzed using ANOVA. The statistical significance level was set at 0.05.

Results

Test using MTT assay showed that the viability of all the test groups was above 50%. A normal distribution test using the Shapiro-Wilk showed that the data were normal distribution ($p>0.05$). The homogeneity test using Levene's test indicated that the data were homogeneous ($p>0.05$). Test using one-way ANOVA demonstrated that there was no significant difference between the control group and all the test groups ($p>0.05$).

Group	Optical Density	Osteoblast viable cells (%)
Control	0.392 ± 0.0954	99.99
0.4 mg/ml	0.386 ± 0.0118	97.95
0.2 mg/ml	0.363 ± 0.2043	90.53
0.1 mg/ml	0.369 ± 0.2670	92.68
0.05 mg/ml	0.399 ± 0.1986	102.16
0.025 mg/ml	0.362 ± 0.1222	90.41
0.0125 mg/ml	0.358 ± 0.0954	89.02
0.00625 mg/ml	0.366 ± 0.0351	91.71

Table 1. The percentage of osteoblast viable cells treated with collagen extract from Oshphronemus gouramy scales at concentrations of 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.025 mg/ml, 0.0125 mg/ml, 0.00625 mg/ml and control group.

The percentage of osteoblast viable cells at concentration of 0.4 mg/ml was 97.95%; at 0.2 mg/ml: 90.53%; at 0.1 mg/ml: 92.68%; at 0.05 mg/ml: 102.16%; at 0.025 mg/ml: 90.41%; at 0.0125 mg/ml: 89.02%; and at 0.00625 mg/ml: 91.71%. The highest osteoblast viable cells was found at concentration of 0.05 mg/ml, that is 102.16% (Table 1).

Discussion

Base on the results from FTIR analysis qualitatively in the previous study, collagen extract from Oshphronemus gouramy scales lead to a helical structure. Therefore, collagen extracted from Oshphronemus gouramy scales in this research was categorized into type-1 collagen.[8] The result of the MTT assay showed that collagen extract from Oshphronemus gouramy scales at concentrations of 0.05 mg/ml in the test group has the highest absorbance value, which was shown as OD. This condition demonstrates that the capability of osteoblast cell to proliferation after administration Oshphronemus gouramy scales at concentration of 0.05 mg/ml was the highest. This phenomenon was analogous with the result in previous studies, which was explained that type-1 collagen from freshwater fish could increase the fibroblast cell proliferation.[12,13]

Absorbance value (OD) at concentration of 0.4 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.025 mg/ml, 0.0125 mg/ml, 0.00625 mg/ml was lower than control. This condition demonstrates that osteoblast cell proliferation at these concentrations have lower capability than the control group. However, osteoblast viable cells were still higher than 50%. Base on toxicity parameters of CD50, a substance can be categorized as toxic if the percentage of viable cells after exposure was lower than 50%.[14] This result indicates that collagen extracted from Oshphronemus gouramy scales can be classified as a non-toxic substance.

Osteoblasts are specialized fibroblasts that secrete and mineralize the bone matrix. They develop from mesenchymal precursors. The mineralized extracellular matrix was mainly composed of type 1 collagen and smaller protein but significant amounts of osteocalcin, osteopontin, bone sialoprotein, and inorganic mineral hydroxyapatite. Osteoblast differentiation can be characterized in three stages: cell proliferation, matrix maturation, and matrix mineralization. During proliferation, several extracellular matrix proteins (procollagen type 1)

can be detected.[15-17]

Absorbance value represents the capability of the cell to proliferation. In this study, absorbance demonstrates the number of viable cells. Absorbance value can be influenced by several factors, such as type of solvent, pH, temperature, high electrolyte concentration and the presence of disruptors. High absorbance value reflects the high ability of cell proliferation. Nevertheless, if the cell proliferation rate was too high, it will result in cell death because there was a possibility of changes in cell morphology.[15,18]

MTT was absorbed into living cells then broken down through a reduction reaction by reductase enzymes in the mitochondrial respiration chain into formazan soluble in a purple solvent. DMSO is added to stop the enzymatic reaction and dissolve the formazan so that the purple color of the formazan can be read its absorbance spectrophotometrically with ELISA reader. The absorbance represents the number of viable cells. The stronger the purple color intensity is, the higher the absorbance will be. MTT absorbed into living cells can lead to more formazan formation. This absorbance can be used to calculate the percentage of viable cells as cell response.[6,7]

Statistically, there was no significant difference in the mean of osteoblast cell count between in the group administrated with collagen extracted from Oshphronemus gouramy scales and the group without collagen extracted from Oshphronemus gouramy scales. The result showed that collagen extracted from Oshphronemus gouramy scales can't influence cell growth. Indeed, this material can increase osteoblast cell growth at concentration of 0.05mg/ml. This is similar to previous research conducted by Zheng et al[19], they suggested that type 1 collagen scaffold can support the growth of mesenchymal stem cells and be used for bone tissue engineering. The previous research revealed that type 1 collagen from freshwater can increase fibroblast cell proliferation and can be used in tissue engineering technology.[12,20]

Collagen is considered to be the most promising material for tissue engineering because of its excellent biocompatibility, degradability, low antigenicity. Collagen contains RGD (Arg-Gly-Asp) and non-RGD peptides that can bind to cell surface related to integrin, consequently, collagen can facilitate migration, adhesion, proliferation, and differentiation of the cell. Type 1 collagen was the main component of bone. It has been used as an extracellular matrix (ECM) or scaffolds for bone regeneration, including enhancing osteoblastic differentiation and bone formation.[16,19,21]

The use of collagen in tissue engineering as graft material must have the ability to stimulate proliferation and differentiation cells in the recipient site.[22] In clinical studies, the use of collagen material in wound healing can stimulate the activity of hemostatic material, thus increasing the cell activity by good vascular formation. Collagen has the ability as a biological scaffold for the growth of endothelial cells and cell progenitors derived from periodontal ligament.[23,24] Collagen can stimulate the presence of osteoblastic phenotype mediated by increases in alkaline phosphatase activity.[25] Collagen membrane in combination with Simvastatin result in an increase of osteoprotegerin level in gingival crevicular fluid.[26] Osteoprotegerin was required during bone remodelling.[27] Thus, collagen plays an important role in bone formation. Collagen derived from Oshphronemus gouramy scales was expected to stimulate osteoblast proliferation during alveolar bone regeneration. This capability was required in periodontal tissue regeneration especially for periodontal defect reconstruction.

Conclusions

Collagen extracted from Oshphronemus gouramy scales has high viability against osteoblast cell culture. Collagen extracted from Oshphronemus gouramy scales was not toxic to osteoblast cells, thus it can be used as an alternative biomaterial in periodontal tissue engineering.

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Declaration of Interest

The authors report no conflict of interest.

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