Viability Test of the Gourami Scales Collagen Extract (Osphronemus Goramy) on the Human Gingival Fibroblast Cells

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Abstract

Background: Periodontal disease is a pathological inflammatory condition of the periodontal tissues which surround the teeth, including Human Gingival Fibroblasts (HGF). HGF regeneration is through the accelerating proliferation of tissue engineering therapy needs. Generally, the tissue engineering uses regenerative materials from cow or pig as the therapies, but these materials have some flaws. Thereby, this research aims to find the alternative materials regenerative tissue engineering scaffold collagen type 1 derived from the gourami fish scales. This research is also conducted to test the viability of fish scales collagen gourami against the Human Gingival Fibroblasts for 24 hours.

Objective: To determine the concentration of fish scale collagen gourami which can maintain the viability of human gingival fibroblast cells for 24 hours.

Method: HGF is taken from the healthy gingiva and planted in 96 well plates. Fish scales collagen gourami with a concentration of 0.32 mg/ml, 0.16 mg/ml, 0.04 mg/ml, 0.02 mg/ml and 0.01 mg/ml were added to each well and incubated during 24 hours. MTT Assay is performed to see the viability of fibroblast cells.

Results: The viability of HGF were increased after the addition of fish scales collagen gourami on the concentration 0.32 mg/ml until 0.01 mg/ml. The viability of the cells after the addition of fish scale collagen gourami was shown above 100%.

Conclusion: Fish scales collagen gourami has the potential in tissue engineering and the concentration of 0.01 mg/ml shows the highest viability of HGF.

Keywords: *Collagen extract, gourami scales, human gingival fibroblast cells,MTT assay, viability.*

Introduction

Periodontitis is an inflammation caused by infection of the supporting tissues of the teeth, progressive damage to the periodontal ligament, and alveolar bone [1]. This disease is caused by the induction 90% of facultative anaerobic bacteria and 75% of negative bacteria[2]. Fibroblasts are stem cells that play a role in forming and placing fibers in the matrix, especially collagen fibers. These cells secrete small tropocollagen molecules that combine in the basic substance to form collagen fibers[3]. Periodontal disease is a dental and oral disease with a high prevalence rate. It is also a disease in the oral cavity that affects almost all humans in the world as well as reaches 50% of the adult population[2].

Engineering tissue technology is needed to

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accelerate the regeneration and healing of periodontal tissue. The tissue engineering approaches to bone and periodontal regeneration combines three key elements to improve the regeneration, namely progenitor cells, scaffold or supporting matrix, and molecular signal (growth factor)[4]. Scaffold serves to provide structure and substrate for the tissue growth and will be degraded after that healthy tissue grows. Cells are needed as inductors of cells (fibroblasts) for adhesion, regeneration, and differentiation from the primitive cells to the specific ones needed in the scaffold to form a healthy new tissue. Growth factor functions for biophysical stimuli and keeps cell growth and differentiation in the scaffold[5].

Fish can be used as raw material to produce collagen. Collagen sourced from the skin, and fish bones has a smaller molecular structure compared to collagen made from cows or pigs, so it is easier to absorb[6]. Collagen from the fish scales is derivative collagen from fish, and extracted from the fish scales. Therefore, there is no need to worry about mammalian diseases, such as mad cow disease or bird flu virus[7]. Based on research of Nagai et al., (2004), there are components of fish scales including 70% water, 27% protein, 1% fat, and 2% ash. Organic compounds consist of 40-90% in the fish scales, while the rest are collagen.

Viability is the ability of a cell to survive. The viability test was determined by 3- (4,5-Dimethylthiazol-2-yl) -2,5-diphenyl-tetrazoliumbromide) MTT. This study aimed to determine the concentration of fish scale collagen gourami which can maintain the viability of human gingival fibroblast cells for 24 hours. Human gingival fibroblast cells were used because fibroblast cells were the most important cells in human periodontal tissue[8].

Material and Method

This type of research is a laboratory experiment with the design of The Post-Test Only control group design. This research was conducted in the Stem Cell Research and Development Center, Airlangga University and cthe hemical engineering laboratory, Politeknik Negeri Malang.

The tools and materials used are 200µl micropipette, small test tube, used tube, 96-well plate, cone tube, yellow tip and blue tip, ELISA reader, tweezers, petri dish, incubator, tissue, aluminum foil. Saline phosphate buffer (PBS) 10%, complete media (MK) (α-MEM 500 mL, FBS 10%, Penicillin, Streptomycin 2%, fungizone 0.5%), DMSO 7.5 mL, MTT 5 mg/mL PBS (50 mg MTT and 10 mL PBS), 10% SDS in 0.1 N HCl, Gourami scales, Human Gingival Fibroblast Cells (HGF), and 10% bovine serum albumin (FBS), Tripsin 0.25% as much as 2 mL, Aquades .

The Making of Gourami Scale Collagen Extract: The addition of chelanting agent (EDTA) 1 N for the decalcification process as well as the addition of 0.5 M acetic acid (acid solubility collagen) are conducted. The next one is the addition of pepsin (pepsin solubility collagen) 0.1 gr.Stirred it with an ultrasonic device at 4º C for 18 hours, and then filtered it. The addition of 0.5 M NaCl was then conducted by centrifugation in the small tubes with a speed of 4000 rpm.Afterward,washed with distilled water and then removed (salting out) it. The lyophilization process with a freezedryer to remove the water with a condenser temperature of -76ºC and ambient temperature of 23.6ºC for 12 hours until the water runs out[9].

The Stage of Fibroblast Cell Management: The gingival tissue was washed for 3 times with PBS containing penicillin and streptomycin antibiotics in order to avoid the possibility of bacterial contamination. Afterward, it was cut to approximately $1mm^3$ and covered with deckglass. The collagenase was added later on for 30 minutes at 37ºC. The tissue was then washed and centrifuged for 6 minutes. The cells obtained were cultured with a growing and incubated medium in a 5% CO_2 incubator with a temperature of 37ºC for 3 days. Moreover, it was observed every day until the cells are confluent. In addition, the culture medium is replaced every three days until the cell is confluent and passages are performed.

The Stage of Fibroblast Cell Culture: Primary human gingival fibroblast cell culture in the Alpha Modified Eagle's Medium (αMEM). Culture was added with 150 µg/ml Fetal Bovine Serum (FBS) 10%, 10 µg/ml Fungizone 0.5%, 100 µg/ml 2% Citrate. Afterward,the confluent cells dipasase to be propagated. The cell medium is removed and washed with PBS. The next one, that cell is released with a 2 ml trypsin enzyme and incubated safely 5 minutes at 37oC and 5% CO2. After that cell is removed, then added a stopper and resuspended, centrifuged 25000 rpm for 6 minutes. Finally, pellets are planted on a 10 cm plate with αMEM medium.

Harvesting Cells: The human gingival fibroblast

cells were taken from CO_2 incubators and then cell conditions were observed (80% confluent cell cultures were used for harvesting). Those cells were harvested according to the harvest protocols. In addition, the cells are seen using a microscope. Finally, the container is tapped so that the cell is floating.

The Stage of Treatment and Readings: The microplate with concentrations of 0.32 mg/ml, 0.16 mg/ml, 0.04 mg/ml, 0.02 mg/ml, 0.01 mg/ml. well the last microplate was filled with Alfa Modified Eagle's Medium (αMEM) culture, Fetal Bovine Serum (FBS) 10% and Pen-Strep 2% as much as 100µL as media control, The next one, the microplate contained only human gingival fibroblast cell culture and αMEM culture because it is used as cell control. The microplate is removed from the incubator. The 20 µl of MTT solution are added to each and finally is well ready to be used[10].

Results

% life cells: According to the results of reading OD (Optical Density), the average results of the research shown in table 1 are as follows.

Tables 1: Absorbance results in the treatment group gourami scale collagen extract for 24 hours.

Treatment	N	Mean
Media Control	4	0,090
Cells Control	4	0,634
Concentration 0.32 mg/ml	4	0,721
Concentration 0.16 mg/ml	4	0,760
Concentration 0.04 mg/ml	4	0,766
Concentration 0.02 mg/ml	4	0,771
Concentration 0.01 mg/ml	4	0,840

Based on the results of the study, it can be seen that the average value of the absorbance of the cell control group for 24 hours is 0.634. The lowest absorbance of theextracts group of gourami scales collagen at a concentration of 0.32 mg/ml of 0.721. The average absorbance of theextracts group of gourami scales collagen was highest at a concentration of 0.01 mg/ml of 0.840.

Tabels 2: Vibility results in the treatment group gourami scale collagen extract for 24 hours.

Concentration	Percentage of Live Cells (%)
0.32 mg/ml	115.97%
0.16 mg/ml	122.99%
0.04 mg/ml	124.23%
0.02 mg/ml	125.06%
0.01 mg/ml	137,82%

Statistical Analysis: It can be concluded that the data are normally distributed. Afterward, from the homogeneity testing using Levene's Test with Sig. >0.05can be concluded that the data is homogeneous. In addition, the statistical tests were carried out using ANOVA One Way at the significance level of Sig. <0.05 and significant differences were obtained. It then continued by performing multiple comparisons using post hoc test Tukey HSD which concluded that all groups had significant differences except the concentration group control cell against 0.16 mg/ml and 0.32 mg/ml.

Discussion

Periodontal tissue has many important constituent components, such as collagen and fibroblasts. Fibroblast cells function as producers of connective tissue. The coats produced by fibroblast cells include collagen fibers, reticulum fibers, oxytalan fibers, and elastic fibers. Fibroblast cells secrete large amounts of protein, such as collagen, and abnormal collagen deposition in which it is a feature of the scarring process involving TGF-β1 and PDGF as a migratory fibroblast medium[11]. Fibroblast cells secrete cytokines and several growth factors (growth factors) which can stimulate cell proliferation and inhibit the process of differentiation[12].

Proliferation of fibroblasts can be accelerated by giving tissue engineering. Tissue engineering functions as returning, regenerating, maintaining, or improving defective or lost tissue function[13]. One type of engineering tissue is scaffold. Scaffold is a porous solid biomaterial that functions as (1) promoter of interactions between cells and biomaterials; cell adhesion; extracellular matrix deposits; migration media and fibroblast cultures; as well as niches for stem cell proliferation (2) supplying gas for cell survival, proliferation, and cell differentiation, (3) can adjust to the rate of tissue regeneration, (4) and relieve the inflammation[14,15].

The basic principle is the work of mitochondrial enzymes in active cells that metabolize tetrazolium salts, so that the tetrazolium ring is broken down by the dehydrogenase enzyme which causes the tetrazolium to turn into an insoluble and purple formazan. The color change of tetrazolium salt is caused by a decrease in the metabolic activity of cells that form NADH or NADPH. This purple color will be measured by absorbance.

Statistically, the comparison of the cell control group with the concentration group of 0.04 mg/ml, 0.02 mg/ml and 0.01 mg/ml experienced a significant value. Meanwhile,the concentration of 0.32 mg/ml and 0.16 mg/ml did not have a significant value. This shows that the plateau effect occurs at exposure to concentrations of 0.16 mg/ml and 0.32 mg/ml, so that exposure to these concentrations does not significantly affect cell proliferation. The research data is in accordance with the loading-dose theory of drugs, namely the higher the dose given, the therapeutic effect on target cells is no better than the lower dose[16].

Scaffold composition consists of polymer poly (alpha-hydroxyl) which can be degraded in the body (PLA, PGA, PLGA) and natural polymers. Natural polymers are divided into proteins (collagen, silk, fibrinogen, elastic, creatine, or actin), polysaccharides (cellulose, amylose, dextran, chitosan (chitosan), and glycosaminoglycans), and polynucleotides (DNA and RNA)[17].

Among the various types of scaffold that has been mentioned, collagen-based scaffold (mainly type I) is widely used. This is because it has more advantages than the other scaffold constituents both natural and synthetic, including those identical to the extracellular matrix, adaptable in various forms, biocompatible, having biodegradation power, increase proliferation, and trigger cell differentiation[18,19].

Fibrillar type I collagen was chosen because it is the gold standard in the formation of biomaterials and is a type of collagen that is widely found in the human body – 90%. The telopeptidesfound in the collagen scaffold can cause mild inflammatory reactions that can affect the regeneration process[15]. Cells interact with scaffold through the Arg-Gly-Asp (RGD) ligand that is similar to the natural extracellular matrix on its surface[20]. The biodegradation properties of collagen scaffold can trigger the restoration of tissue structure and function. The degradation of collagen scaffold based on the type I collagen to type III by MMP-9 will induce the attraction of chemotaxis from human fibroblasts[21].

Collagen derived from gourami scales can be an alternative material consideration for the tissue engineering because of its abundant collagen content and good biocompatibility[22].

Conclusion

Collagen extract of gourami scales was viable on Human Gingiva Fibroblast cells, with the highest yield of viability at a concentration of 0.01 mg/ml is 137.82%.

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