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Effect of Collagen-Chitosan-Glycerol Composition in Scaffold for Gingival Recession Therapy

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Abstract. The case of gingival recession has a high prevalence, about 88% of the population of the United States in one or more locations suffering from gingival recession. One of the handling cases of gingival recession is to use scaffold that includes the development of tissue and cell engineering. This study aims to determine the best composition variation scaffold of collagen-chitosan with the addition of glycerol. The process of synthesis of collagen-chitosan-glycerol scaffold using freeze dry method that can form pores on the scaffold. Characterization was also carried out on the results of the synthesis of collagen-chitosan scaffold with the addition of glycerol include the morphological characterization, tensile, cytotoxicity, swelling, degradation, and thickness. The results of morphological characterization showed pore size ranged from 26.68 - 191.7 μm with a thickness of 0.51 - 0.65 mm which was suitable for handling of gingival recession cases. The result of tensile test showed that the variation of 9: 1 has the lowest value that is 2.87 MPa where the value is close to tensile strength value for periodontal which has a value ranging from 2.75 to 5.13. The characterization of cytotoxicity shows a value that is less in line with the literature, where live cells <50%. This is because collagen and chitosan have an acidic pH so that the cells cannot reproduce. Characterization of degradation shows all the variations experienced a severe reduction process from day to day. The characterization of the swelling of all samples was equilibrated at 7 minute. Chitosan-collagen scaffold with the addition of glycerol has good potential as a scaffold candidate for gingival recession therapy based on morphological characterization (thickness and surface structure), the mechanical strength (tensile strength), degradation, and the degree of swelling.

Introduction

Gingival recession is a condition characterized by the displacement of the gingival edge position apically from the cementoenamel junction (CEJ), because of the loss of alveolar bone attachment tissue which results in the opening of the tooth root surface. This gum recession can be local or comprehensive, depending on the cause. According to the United States National Survey at over 65 years old (elderly) by 88% and ages 18-64 (adults) by 50% who experience gingival recession in one or more locations, increasing age can increase the frequency of progressive recessions. Gingival recession is observed in adults otherwise free of periodontal disease with a high standard of oral hygiene [2]. Recession is considered as an esthetic problem. Nowadays, the fields of esthetic dentistry and orthodontics have been concerned with the role that gingival architecture plays in dental esthetics [3]. Based on existing problems, handling cases of gum recession with surgery is quite high. Surgery can cause psychological trauma to the patient. The possibility of more gingival recession is quite high because individuals do not maintain oral hygiene so that the root surface of the tooth that was originally closed will open again [4].

It is necessary to develop natural materials or suitable synthetic materials as an alternative to handling cases of gingival recession. An evidence has shown that natural materials can resemble an extracellular matrix (ECM) and have biocompatibility, biodegradability, and biological functions that can make them suitable for tissue engineering applications [5]. Scaffold is a 3D template that

helps accelerate the formation of new tissues, this is due to the material used by collagen and chitosan in accordance with the nature of body tissues [6]. Collagen and chitosan have great potential in designing various bioactive ingredients for different biomedical purposes [7].

Collagen is a natural polymer that has three-quarters of it dry weight of the skin and the most common component of the extracellular matrix, bone, tendons, ligaments, and cartilage of mammalian tissues [8]. Collagen has the ability as a biological scaffold for cell growth endothelium and progenitor cells from the periodontal ligament. On scaffold based, collagen has been widely used in periodontal therapy and implants as barrier to prevent epithelial cell migration and encourage re-release by cells with regenerative potential [9]. However, collagen scaffold often loses shape and size due to its degradation fast when in direct contact with body fluids. Collagen is a polymer nature tends to be easily degraded because it has amino acids one of them is proline which is prone to react when it is exposed to thermal spontaneous denaturation when exposed to body heat and no triple helix is formed [10]. In making scaffold for the treatment of gingival recession, collagen will be synthesized with chitosan.

Chitosan is a material that has a longer degradation rate compared to collagen, due to steric or steric hindrance obstruction will make the position between the molecules more tightly. Chitosan is a copolymers of 2-glucosamine and N-acetyl-2 glucosamine, derivative compounds from chitin which is environmentally friendly, including biodegradable, biocompatible, biofunctional and bioadsorbable [11]. Chitosan including natural polymers that easily become gels in environments with pH low but has a fragile and easily torn. Chitosan has potential high in tissue engineering applications. Application of chitosan material on scaffolds for periodontal namely that the results of the scaffold mechanical properties increase. One of the most important benefits of chitosan is high bioactivity, which making this material very interesting to be developed into medical applications teeth [12].

Plasticizer is a low molecular weight organic material added to the making of scaffold and has a considerable influence in making scaffold, because it has the function of increasing flexibility and extension of scaffold. The way the plasticizer works is by attaching it between polymer chains. Glycerol is an economical plasticizer, the source is easy to get, can be updated, and is environmentally friendly because easily degraded by nature [13]. Plasticizers are used in the preparation of the membrane/film in order to promote the desired mechanical characteristics. The commonly used plasticizer is glycerol. This study aim is to determine the best composition variation scaffold of collagen-chitosan with the addition of glycerol. Based on the known characteristic of glycerol, in this study, plasticizer glycerol (Gly), were tried in collagen - chitosan films and were evaluated to explore its effect in increasing mechanical strength related with their composisition.

Materials and Methods

Materials and Tools

The material used in this study is collagen from Indonesian Biochitosan products, chitosan from Sigma Aldrich products, 2% glycerol, acetic acid, citric acid, Phosphate Buffer Saline (PBS), and distilled water.

The synthesis equipments are analytical balance, glass beaker, magnetic bar, magnetic stirrer, freezer, lyophilizer, measuring cup, spatula, stopwatch, filter paper, aluminum foil. The characterization equipments are Tensile Strength (Imada HV-500NII Autograph), MTT Assay (Elisa reader), Scanning Electron Microscope (inspect S50, FEI Corp., Japan), Frezee Drying (OHRIST BETA 1-15, Germany).

Collagen-Chitosan-Glycerol Scaffold Synthesis

Scaffold preparation begins with dissolving collagen and chitosan in the form of powder into the recruiters, namely acetic acid and citric acid. After mixing using stirerr for 6 hours until homogeneous with the absence of sediment in the solution if it is left to stand. Then the next process

synthesizes collagen with chitosan in the ratio (v / v) 6: 4, 7: 3, 8: 2, 9: 1 and 2 ml of glycerol 2 ml is added to each variation and stirred until homogeneous. The solution is poured into a petri dish to make the lyophylization process.

Morphological Test

The test was carried out using a scanning electron microscope (SEM), so that it could determine the surface morphology and cross section of collagen-chitosan scaffold samples with the addition of glycerol. By doing this SEM test, you will see the pore size and thickness formed from the scaffold. Before the sample is inserted into the scanning electron device, cutting preparation is carried out according to the holder and then coated with Au or Pd using sputtering coating method [14].

Mechanical Strength Test

Scaffold is cut according to the test standard and both ends are linked to a tensile test so that the mechanical strength of collagen-chitosan-scaffold can be measured by the addition of glycerol [15]. From the treatment will be obtained a quantity that is used as a reference of the mechanical strength of the sample.

$$\sigma = \frac{F}{A}$$

Information: σ = Stress (N/m^2)
F = Load (N)
A = Surface Area (m^2)

Cytotoxicity test (MTT Assay)

In this test phase the MTT assay test method is performed using Elisa Reader. BHK-21 cell culture in Eagle media was then transferred to a microwell plate with 100 µl of each well and incubated for 24 hours. The media was discarded and washed with 250 µl PBS. DMEM was given as much as 30 µl and MTT solution [3- (4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide] as much as 10 µl and then allowed to stand for 3 hours and thrown back. 10 µl of DMSO solution was added to the microwell plate and dishaker for 5 minutes to mix with a homogeneous DMSO solution. Many percentages of living cells in the scaffold can be calculated:

$$\text{Percentage of Living Cell (\%)} = \frac{\text{OD Treatment Cell} - \text{OD Control Media}}{\text{OD Control Cell} - \text{OD Control Media}}$$

Information: % Living Cell = percentage of living cell count after testing
OD Treatment Cell = formazan optical density values for each sample after treatment
OD Control Media = formazan optical density values in control media
OD Control Cell = formazan optical density values in control cell

Swelling test

The ability of swelling scaffold was determined by subduing or soaking scaffold at normal pH in phosphate buffer saline (PBS) at room temperature. The wet weight of the scaffold was calculated for 1 to 7 minutes and dried using a sponge filter paper to remove water absorbed on the surface and then immediately weighed. Previously, collagen-chitosan scaffold with the addition of glycerol was cut into small pieces with a size of 1 cm x 1 cm in square shape. Many absorptions that occurs in the scaffold can be calculated:

$$Esw\ (\%) = \frac{we - w0}{w0}\ x\ 100\ \%$$

Information: Esw = persentase swelling scaffold
Wo = initial weight before submersion
We = sample weight when submersion

Degradation test

The degradation test of the scaffold can be done by soaking the sample in Phosphate Buffer Saline solution with pH 7. Samples of collagen-chitosan scaffold with the addition of glycerol plasticizer are formed with a size of 1 x 1 cm, then put into a container containing 15 ml PBS solution and closed meeting and stored at 37ºC for 7-14 days to observe degradation in scaffold samples [16].

$$D\ (\%) = \frac{Wo - W_t}{W_o} \text{ X } 100\%$$

Information: D(%) = percentage weight
W_t = the final weight of the scaffold after soaking on PBS
W_o = initial weight in dry conditions

Results

Morphological Test Results

The first characterization is morphologically tested by scanning electron microscopy (SEM) to determine the surface structure of the sample. The magnification made is 300X, as seen in Figure 1.

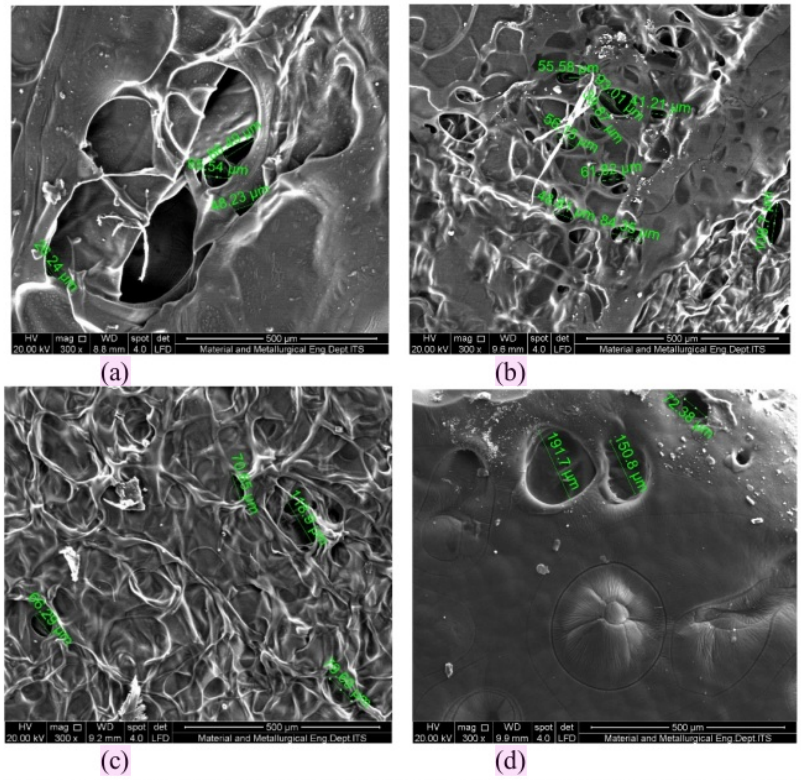

Figure 1. Observations with Vertical Cutting in Scaffolds for Thickness Measurement Results (magnification 300x): (a) 6: 4: 2; (b) 7: 3: 2; (c) 8: 2: 2; (d) 9: 1: 2.

Cytotoxicity Results

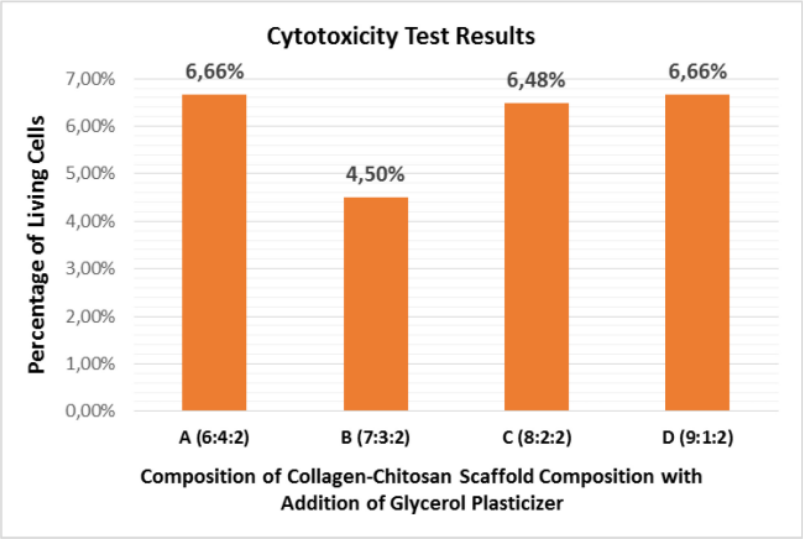

Figure 2. Graph of Percentage of Living Cells from Collagen-Chitosan-Glycerol Scaffold Samples.

Swelling Test Results

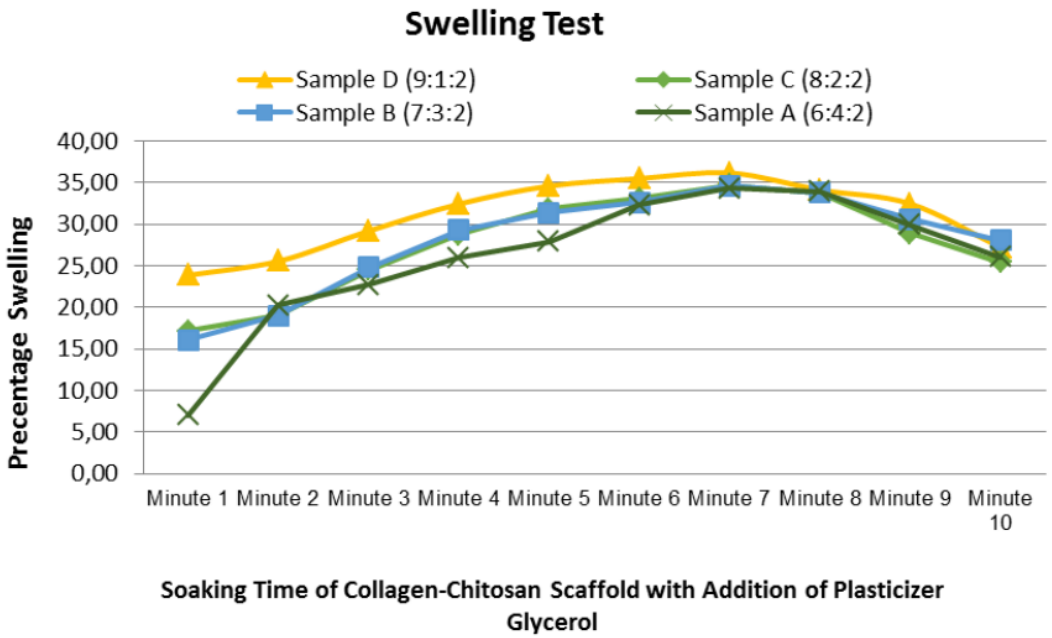

Figure 3. Graph of Swelling Percentage of Collagen-Chitosan-Glycerol Scaffold.

Degradation Results

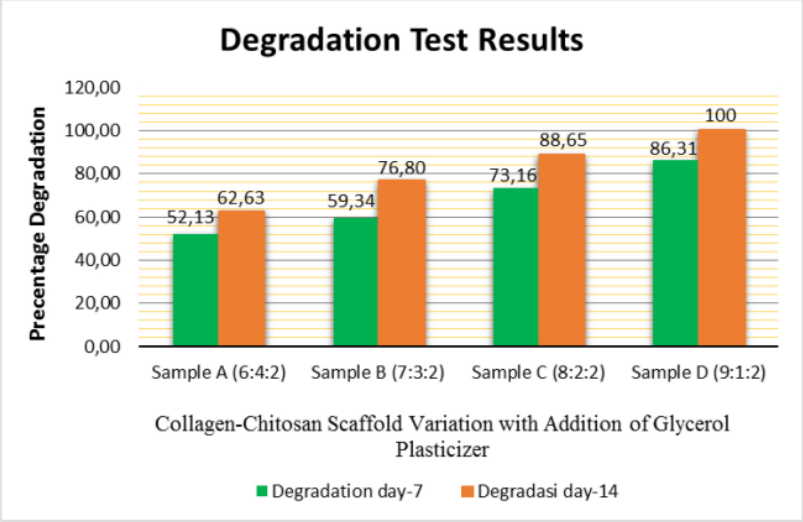

Figure 4. Percentage Degradation Chart of Collagen-Chitosan-Glycerol Scaffold.

Discussions

In this study samples were carried out variations in the composition of collagen-chitosan with a ratio of 6: 4, 7: 3, 8: 2, and 9: 1 then each variation was added with 2 ml of glycerol.

The thickness produced from the scaffold ranges from 0.51 to 0.65 mm, so that from that day the thickness produced is close to thickness according to Peng et al [17] which is 0-2 mm. Whereas for pore sizes obtained the range between 66.29 - 191.7 µm. Where this pore size has entered into the application for periodontal. According to O'Brien et al [18], that for periodontal case applications it takes 63-150 µm pores. The presence of pore formation is needed for cell nutrition, proliferation, and migration of blood vessel-forming tissue and the formation of new tissues [19].

Cytotoxicity test aims to determine the toxicity value of a material directly to cell culture [20]. The cells used are Baby Hamster Kidney-21 fibroblast cells (BHK-21) because these cells originate from the embryo so easy to grow and easy to do sub-culture [21]. In this test there is a specimen which contains media controls and cell controls. Media control is a mixture of eagle media while cell control consists of BHK-21 fibroblast cells and Eagle's. The results of the calculation of each sample that has been through the process of cytotoxicity and reading through Elisa Reader. The four scaffold samples show values that are below the toxicity standard of a sample. This is indicated by the percentage of living cells from the four scaffold samples that showed values below 50% with a range of percentage of living cells, namely 4.50 - 6.66%. This value is not in accordance with what is expected, because a material is said to be non-toxic if the percentage of living cells is stated to be more than 50% [22]. Low pH levels in acids can cause cells to denaturate proteins, namely damage to intramolecular covalent disulfide bonds with ionic bonds, hydrophobic bonds, and hydrogen bonds [23].

Swelling test is carried out to find out how the scaffold response to the solution environment is around. This test uses PBS as an immersion medium. All samples have reached equilibrium after being immersed for 7 minutes into Phosphate Buffer Saline solution. Equilibrium occurs when the sample has experienced a saturation phase in a solution of PBS solution. The saturation phase occurs when the sample is unable to absorb the PBS solution optimally. Decrease in swelling percentage occurs at 8, 9, and 10 minutes because at that minute the sample has been damaged. This is due to the interaction of hydrogen that occurs between collagen and chitosan in that minute during the process of soaking PBS sample solution has exceeded the maximum limit for binding water.

Collagen can act as a modifier of chitosan surface which can cause the value of swelling to be high [17].

Degradation test is used to find out how long the sample can break down in the body, so that the cell can then produce an extracellular matrix [18]. The degradation test was carried out as a simulation when the scaffold was applied to the gingiva. Based on the graph in Figure 4, it appears that collagen-chitosan scaffold with the addition of glycerol plasticizer for sample D (9: 1: 2) experienced a degradation percentage of 100% at day 14. A study said that healing for gingival application took 7-14 days to form gingiva perfectly [24]. It can be said that the more collagen composition in the scaffold causes the faster degraded time. Collagen has the ability to easily lose shape and size due to rapid degradation when dealing with body fluids or cell culture media [17].

Conclusion

Based on the observation data, it can be concluded that the scaffold results from SEM test showed that the sample has pore size which meets the standards for periodontal applications. Cytotoxicity test results show that all four samples have a percentage of live cell values below 50% due to the low pH. Macroscopic characteristics of scaffold on tensile strength test results are showed that the addition of collagen composition can reduce the tensile strength value. The results of the swelling test are showed that all samples experienced an equilibrium point when absorbing PBS solution in the 7th minute. The results of the degradation test are showed that samples degraded for 14 days were obtained in sample with collagen ratio: chitosan: glycerol ie 9: 1: 2 with a value 100%, which is in accordance with the degradation of scaffold in gingival application. Whereas, the thickness test results are show that all samples have thickness measurement that meets gingival applications with a standard gingival thickness of 0-2 mm.

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