The Chitosan-chicken Shank Collagen Used as Scaffold Through Lymphocyte Cell Proliferation in Bone Regeneration Process

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ABSTRACT

Introduction. Alveolar bone defect in dentistry can be caused by injury after tooth extraction, periodontal disease, enucleation of a cyst, and tumor surgery. Scaffold in tissue engineering is an important material that can stimulate osteogenesis process. Lymphocyte cells have a role in promoting and accelerating the proliferation of supporting cells like osteoblast to accelerate the bone regeneration process.

Objective. The purpose of this study was to determine the effect of chitosan-collagen chicken shank collagen used as scaffold for bone regeneration through lymphocyte cell proliferation.

Method. Twelve Wistar rats *(Rattus norvegicus)* were prepared as animal models in this study. Bone defects are intentionally made in both the right and left femur bones of the rat. Total samples were 24 divided into four groups: Group 1 as a control Group using 3% CMC-Na, Group 2 using chitosan scaffold only, Group 3 using chitosan-chicken shank collagen scaffold (50:50), and Group 4 using chitosan-chicken shank collagen scaffold (80:20). The animals were sacrificed on the 5th day, and histopathological examination was carried out to observe the number of lymphocyte cells.

Results. Significant differences between all groups can be showed in the one-way ANOVA test (p value>0.05). The highest lymphocyte cells were found in Group 3 with chitosan-chicken shank collagen scaffold (50:50).

Conclusion. The chitosan-chicken shank collagen used as scaffold can increase the bone regeneration process through increased lymphocyte cell proliferation.

Keywords: chitosan, chicken shank collagen, scaffold, lymphocyte, bone regeneration

INTRODUCTION

Alveolar bone damage in dentistry cases can be caused by periodontal disease, major trauma after tooth extraction, post enucleation cysts, and post-surgery tumors.[1] Periodontal disease is the second most common dental health condition in Indonesia, after dental caries, a problem in society. The prevalence of periodontitis in Indonesia reaches 37,057 cases.[2] If periodontal disease is not treated immediately, bone tissue defect will result in tooth loss.[3,4] Cases of bone defect involve damage to the cancellous and cortical bone.

One biomaterial which can increase bone regeneration process requires bone graft material. A bone graft consists of three types, namely, autograft, allograft, and xenograft. Autograft is currently known as the gold standard of

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treatment for bone defects. This graft requires additional surgical procedures that take longer, causes high morbidity rates, and raises the risk of infection.[5] In recent years, with bone regeneration innovation, biomaterials focused on designing physicochemically suitable scaffolds for cell attachment, proliferation, differentiation, and formation of specific organ tissues.[6] The ideal conditions for making scaffold include osteoconductive, osteoinductive, osteogenic[7], biodegradable, suitable microstructure, and good mechanical properties. Also, the most critical requirement is the ability to stimulate cell adhesion and maintain tissue function.[8]

Some polymer materials that have been developed in tissue engineering include polyglycolic acid (PGA), polylactic acid (PLA), poly (lactic-co-glycolic acid) (PLGA), chitosan, and collagen.[9] Chitosan is widely used as a polymer in tissue engineering and is an amino polysaccharide (poly-1,4-D-glucosamine).[10] This polymer has been considered a material with many functional advantages because it has high biocompatibility, low toxicity, and biodegradable.[5,11] However, a single material made of scaffold using organic materials is less able to meet all the criteria needed in bone tissue regeneration success. The presence of chitosan itself is not enough osteoconductive, so forming new bone is not optimal. Pure chitosan produces low mechanical strength, so that this material must be combined with other materials that can increase mechanical strength. Good mechanical strength will promote osteogenic cell proliferation.[12] The approach to overcoming this weakness is to design a composite by combining the strengths with collagen as an organic material that also plays a role in tissue engineering.[13]

Collagen is one of the most fibrous proteins found in vertebrates, around 25-35% of the total body protein.[14] The collagen sources can be found in the skin, intervertebral discs, bones, cartilages, blood vessels, tendons, significant components of the extracellular matrix, and ligaments.[15] One source of collagen can be found in chicken shank. Chicken shank can be used as an alternative to collagen, which can be utilized in biomedical applications because it is easy to obtain and is affordable.[16] Collagen contains RGD (Arg-Gly-Asp) and non-RGD, which can bind to surface cells related to integrins to facilitate migration, attachment, proliferation, and cell differentiation.[15] Collagen has been used in tissue engineering applications because it has good biocompatibility, is biodegradable, and has low antigenicity properties.[17,18] Collagen can be made into a scaffold for bone regeneration because it can be absorbed by the body and has an excellent attachment to cells.[19] Biodegradation time of collagen is fast, and this material has a weak mechanical strength. A combination of two polymer materials is needed to improve scaffold potential for tissue engineering to cover these weaknesses. A variety of chitosan and collagen can form a unique structure that can increase mechanical strength and reduce collagenase's biodegradation level.[20,21] In the previous study, the combination of chitosan and chicken shank collagen as scaffold can improve the regeneration process of bone defect in *Rattus norvegicus* animals on the 14th day (by increasing RANKL expressions and decreasing osteoclast cell numbers).[22]

Bone healing is characterized by a series of cellular, molecular, and tissue transformation processes consisting of resorption and soft and hard tissue formation. This process starts from the inflammatory stage with inflammatory cells such as macrophages and lymphocytes to the bone remodeling stage.[23] Lymphocyte cells play a significant role in the body's immune response or protection system.[24] The maximum presence of lymphocytes can be seen on the 5th day during the healing process.[25]

Based on the description described above, the researchers combined two organic natural ingredients, namely, chitosan and chicken shank collagen, into a scaffold to determine the effect of chitosan-collagen shank through lymphocyte cell proliferation in the bone regeneration process.

MATERIALS AND METHODS

This is an experimental laboratory study with a post-test only control group design. The fabrication process of chicken shank collagen and making the combination of chitosan and chicken shank collagen scaffold was carried out at the Research Service Unit (ULP), Faculty of Pharmacy, Airlangga University and Laboratory of Human Genetics, Tropical Disease Center of Universitas Airlangga. Treatment in experimental animals was conducted at the Laboratory of Biochemistry, Faculty of Medicine, Universitas Airlangga. Histopathological preparation was carried out at the Diagnostic Center, Dr. Soetomo Hospital, Surabaya.

Gel preparation

The gel base of the control group was made of Natrium-Carboxy Methyl Cellulose (Na-CMC) at a concentration of 3%. This study's chitosan brand name was SIGMA ALDRICH with a >75-85% deacetylation degree.[22] Chitosan gel was made with 200 mg chitosan powder mixed with 5mL (0.1M) acetic acid added with 15mL (0.1M) NaOH. The mixture was centrifuged at 9000 rpm to obtain pure chitosan gel. Collagen was obtained from a chicken shank collagen extract. The chicken shank in this study was purchased from PT. Wonokoyo and certified healthy. The chicken shank was cut into small pieces and mashed. The mashed chicken shank was mixed with 250 U/mg of the enzyme trypsin powder and then incubated at 37°C for 24 hours. Glacial acetic acid was added. The preparation was stored at 4°C for 48 hours. The preparation was mixed to form fibers and centrifuged to obtain the supernatant. They are centrifuged twice to get pure supernatants. The supernatant obtained was mixed with 0.5M acetic acid, and 5% NaCl was added to form a collagen band. The process was repeated three times. The collagen tapes were filtered using filter paper. Collagen was dialyzed and centrifuged again.

Scaffold fabrication

The chitosan and collagen scaffold fabrication was done by mixing chitosan gel and chicken shank collagen gel homogeneously with ratios of 50:50 and 80:20. Next, the gel was inserted into the Teflon-made scaffold mold, was frozen at -20°C for 2 hours, and freeze-dried for 24 hours. The scaffold was then sterilized using a UV clean bench.

Animal handling

In this study, researchers used 12 male Wistar rats (*Rattus norvegicus*) weighing 250-300 mg. Their left and right femur bones were defective. Thus, the total sample used in this study was 24. The samples were classified into four groups, consisting of six samples each. The research groups were namely group 1 as a control group (3% CMC-Na), Group 2 using chitosan scaffold only, Group 3 using chitosan-chicken shank collagen scaffold (50:50), and Group 4 using chitosan-chicken shank collagen scaffold (80:20). Femur bone defect then was made with a diameter and height of 3 mm using a low-speed round bur under anesthesia using ketamine and xylazine. The scaffold material was applied to the defect bone following the treatment of each group.[22]

Ethical clearance for this study was obtained for handling experimental animals from the Dentistry Faculty, Universitas Airlangga (certification number: 41/KKEPK. FKG/IV/2015).

Histopathological preparation

On day 5, bone defects in the control and treatment groups were taken under inhalation anesthesia. The bones were processed for histopathological preparation by staining with hematoxylin-eosin (HE) to determine the proliferation of lymphocyte cells measured manually on five visual field examinations using a 400x magnification light microscope.

Data analysis

This study's data were analyzed by one-way ANOVA test, followed by Tukey HSD to determine the control group's effect, chitosan scaffold, chitosan-chicken shank collagen 50:50, and chitosan-chicken shank collagen 80:20 scaffold on the bone defect on the 5th day, through lymphocyte cell number.

RESULT

We obtained the mean value of lymphocyte cell numbers in the healing process of rat femoral bone defects in the control and the treatment groups using chitosan scaffold, chitosan-chicken shank collagen scaffold 50:50, and chitosan-chicken shank collagen scaffold 80:20. The mean value of lymphocyte cells number observed on day 5 in each group can be seen in Table 1.

Table 1 shows that the highest number of lymphocyte cells was found in the defect treatment group given chitosan-collagen scaffold material 50:50. The second number was seen in the group given chitosan-collagen 80:20 scaffold; the third was seen in the scaffold group chitosan. The smallest number of lymphocytes can be observed in the control group.

Table 1. The mean value of the lymphocyte cells number observed on day 5

Group	∑ Sample	Lymphocyte cells number
Control group	6	9,3333
Chitosan scaffold	6	13,1667
Chitosan-chicken shank collagen scaffold 50:50	6	25,8333
Chitosan-chicken shank collagen scaffold 80:20	6	23,0000

**Based on ANOVA one-way test: the significance value of 0.000 (p <0.05) was obtained*

The following slides are from the histopathological examination by observing the number of lymphocyte cells in the control and treatment groups using a 400x magnification light microscope (Figure 1).

The lymphocyte cell morphology is small, oval, or round with one cell nucleus (purple color); the cytoplasm is light blue and has no granules. The figure above shows that the combination of chitosan and chicken shank collagen scaffold can increase the number of lymphocyte cell numbers in rat femur bones' healing defects. The chitosan-chicken shank collagen scaffold 50:50 is more effective in increasing the number of lymphocyte cells than the 80:20 combination.

The Kolmogorov-Smirnov test statistic was used to test the data's normality to be eligible for the ANOVA one-way test. In the Kolmogorov-Smirnov test, the entire study group's results were obtained with a significance value greater than 0.05 (p> 0.05), which means that all study groups' data were normally distributed. This study's homogeneity test results showed a significant effect of 0.147 for lymphocyte cells and 0.108 (which means to be homogeneous, p> 0.05).

The subsequent analysis is the ANOVA one-way test to determine the significance value of calculating the number of lymphocyte cells. A significance value of 0,000 (p <0.05) was obtained, which means a significant difference between the control group, chitosan scaffold, chitosan-chicken shank collagen scaffold 50:50, and chitosan-chicken shank collagen scaffold 80:20. Although a substantial difference has been found between the four groups, the researchers needed to know which group differed most using the Post Hoc Test Tukey HSD. Based on the resulting test, there are significant differences in the number of lymphocyte cells between one group and another (p <0.05).

DISCUSSION

Currently, bone tissue engineering innovation is focused on applying biomaterials that are being developed, including the scaffold. 3-dimensional (3D) shaped scaffolds with porous structures are suitable for cell adhesion, proliferation, differentiation, and specific tissue formation.[6] Biomaterials

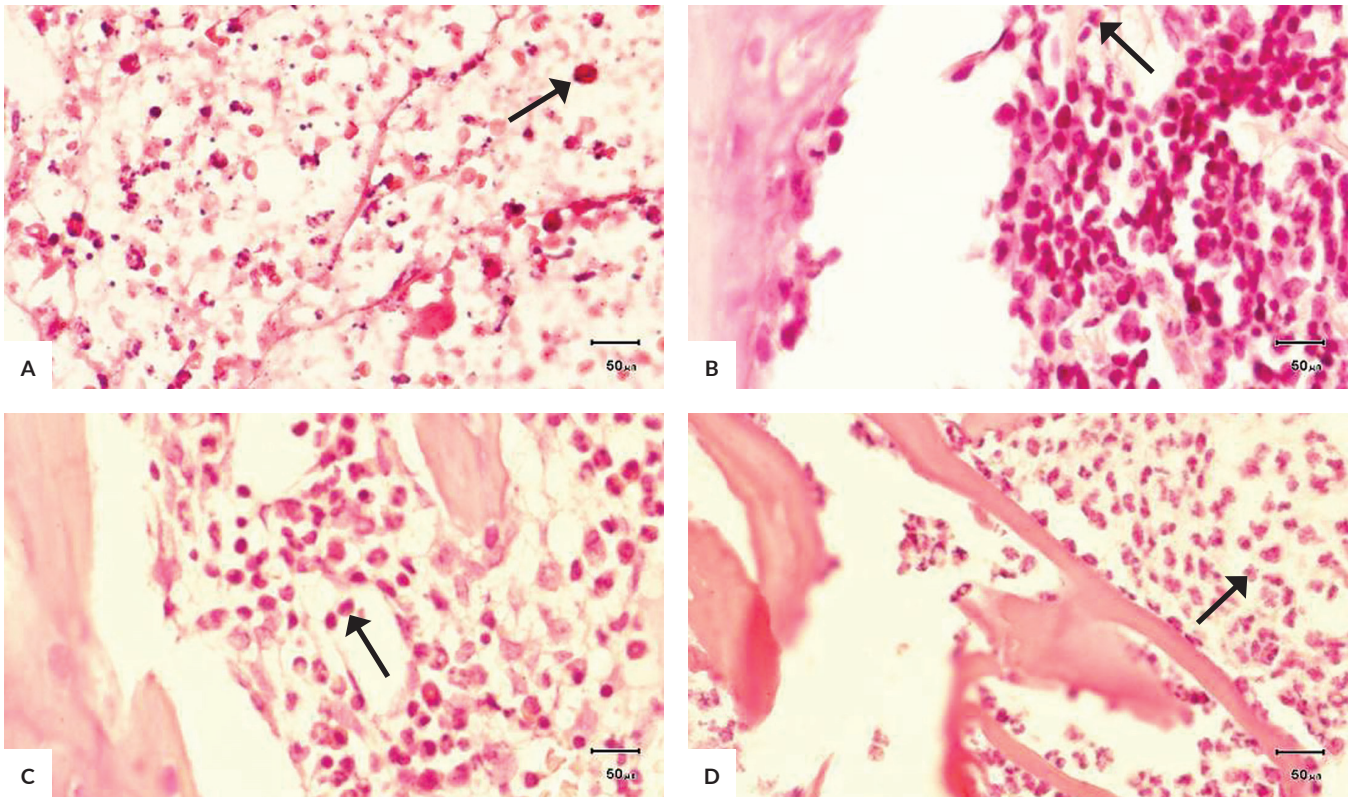

Figure 1. Results of histopathological examination with hematoxylin-eosin (HE) staining by observing the number of lymphocytes cells with 400x light microscope magnification. **(A)** control group; **(B)** chitosan scaffold; **(C)** Chitosan-chicken shank collagen scaffold 50:50; **(D)** Chitosan-chicken shank collagen scaffold 80:20.

that can be made in the form of scaffold include chitosan and collagen.[26] Chitosan and collagen are polymers with good biodegradable and biocompatible properties and low toxicity, so it is suitable to be combined in the form of a scaffold. The conditions of a promising scaffold must have these characteristics.

This study focused on determining chitosan-collagen chicken shank collagen's effect as a scaffold for bone regeneration through lymphocyte cell proliferation on the 5th day.

Chitosan is a natural polysaccharide that is structurally similar to glucosaminoglycan, namely poly-D-glucosamine. In connective tissue, glucosaminoglycans are the most abundant component in the extracellular matrix (ECM).[21] Chitosan is produced from chitin's deacetylation by removing the acetyl group, making this chitosan positively charged (cationic).[27] The higher degree of deacetylation of chitin will give greater cationic properties. Also, the higher the degree of deacetylation will affect the lower the ability of biodegradation.[28] In making scaffold, researchers used chitosan with a degree of 75-85% deacetylation purchased from SIGMA. This positively charged chitosan can form complex ionic bonds by binding to negatively charged materials such as collagen.[29] This chitosan's cationic nature can stimulate cell adhesion as a modulator of cell morphology, differentiation, cell movement, cell synthesis, and function.[30]

Another ingredient used in making scaffolds in this study is collagen. Collagen is obtained from a series of chicken shank synthesis processes. Chicken shank has a fairly high collagen content of around 9.07%.[31] Immunogenic reactions from collagen as bone graft extracted from other species (xenograft) can be eliminated by the trypsin enzyme's role. It is still widely considered acceptable for tissue engineering in other species.[18] In making collagen gel in this study, a chicken shank that has been mashed is processed using the trypsin enzyme to prevent an immunogenic reaction from the chicken shank. A series of processes are carried out to form pure collagen gel.

The fabrication of chitosan scaffold and the scaffold combination of chitosan and chicken shank collagen was done by a series of procedures such as making chicken shank collagen gel, chitosan gel making, freeze-drying process to get 3D and porous scaffold shapes, and sterilization process with UV clean bench tools. The use of UV clean bench sterilization is because chitosan and collagen are not heat resistant, so sterilization is enough to do with radiation. This sterilization is needed to kill bacteria if there are bacteria attached to the scaffold. The scaffold is sterile when applied to the femur bone defect of the Wistar rat.

Hydroxyapatite (HA) has also been extensively studied for biomedical use mainly because of bone composition

similarity. Micro-substituents can alter the microstructure, stability, and crystallinity of the HA structure in implants. Several experimental investigations have shown that substituents can also have extraordinary effects on bone cells that incorporate the implant.[32]

The study results observed that on the 5th day, the average number of lymphocyte cells was highest in the treatment group, given a combination of chitosan and chicken shank collagen scaffold combination 50:50. The second was seen in the chitosan-chicken shank collagen scaffold group 80:20, followed by the chitosan scaffold group. The least number of lymphocyte cells was seen in the control group.

There was a significant difference in the number of lymphocyte cells between groups ($p < 0.05$). Significant differences were also seen when each group was compared ($p < 0.05$). This shows that each group can produce lymphocyte cells in the healing process of rat femur bone defects. The combination of chitosan and chicken shank collagen scaffold makes the most lymphocyte cells compared to the chitosan and control group because the combination of these two materials can form complex ions so that it can increase the mechanical strength of the scaffold and decrease biodegradability[21], can mimic nanostructures from tissues, so that cell adhesion or infiltration occurs better[33] including lymphocyte cells due to collagen material[34], and chitosan[35] can increase IL-10 cytokines produced by lymphocyte cells. High IL-10 cytokines are affected by the high number of lymphocyte cells. In the scaffold group, the chitosan-chicken shank collagen combination can increase the number of lymphocyte cells more than other groups.

During the inflammatory phase, bone defects are infiltrated by adaptive immune cells such as lymphocytes that play a role in bones' healing process.[36] Increasing the number of lymphocyte cells in bone defects can accelerate the healing process of bones. Chitosan-chicken shank collagen scaffold is more effective in producing lymphocyte cell counts in the defect than 80:20 combination chitosan-chicken shank collagen scaffold because, in a previous study by Develioglu (2005), the osteoconductive and osteointegration are related to pore size.[37] The composition of chitosan, which is more significant than collagen, causes the pore size to be smaller so that it takes longer to stimulate cell growth, in this case, lymphocyte cells. Using a 3D scaffold on the bone defects has essential benefits in the treatment group because the scaffold has a microporosity structure that can affect the cellular activity. It can be absorbed by the body such as polymers and speed up network replacement damaged or serves as a framework (extracellular matrix), allowing cells to proliferate and differentiate chondrocyte cells, progenitor cells, etc., that it can maintain network function. Scaffold microporosity affects cellular activities, including stimulating new cell growth, cell adhesion, increasing interface interactions, and supporting cell proliferation so that it will accelerate bone healing.[38-40] This property is vital for biomaterials to direct and encourage the formation of tissue growth. The chitosan scaffold administration shows a smaller number of lymphocyte cells than the combination group because the chitosan scaffold has a rigid nature and is easily fragile, so that bone formation becomes less optimal if it is not combined with other organic materials such as collagen.[8] Collagen has been studied that this biomaterial can stimulate the attachment and growth of cells and tissues. Collagen contains arginine-glycine-aspartate (RGD) and non-RGD that bind the cell surface through integrin receptors so that it has the role of facilitating migration, attachment, proliferation, and cell differentiation.[15,37] Good cell adhesion causes lymphocyte cells to attach to tissue so that it is expected to replace lost bone tissue. The control group that was only given a CMC Na 3% gel produced the smallest number of lymphocyte cells than the treatment group because the gel did not have an active ingredient that played a role in accelerating the bone regeneration.

Chitosan itself as a bone graft material is less osteoconductive, so it needs to be combined with other materials such as collagen to make it more stable.[41] The combination of chitosan and collagen scaffold can be formed a bridge that increases the efficiency of the bonds between the amino acid of the combination of chitosan-collagen chains in the bone tissue. The previous studies showed that a combination of chitosan-chicken shank collagen scaffold could increase the number of osteoblast cells and OPG expression[42] and decrease osteoclast cell number on day 14th Wistar rats bone regeneration process.[25] Both studies follow this research that combination scaffold can increase lymphocyte cells to stimulate and accelerate the proliferation of supporting cells like osteoblast to accelerate the bone regeneration process.

CONCLUSION

Based on the study results, it can be concluded that the combination of chitosan and chicken shank collagen scaffold can increase the lymphocyte cell number in the bone regeneration process on femur bone defects of *Rattus norvegicus*. The application of chitosan-chicken shank collagen scaffold 50:50 is the most effective scaffold to improve the lymphocyte cell number in this study, accelerating bone regeneration.

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Statement of Authorship

All authors participated in data collection and analysis, and approved the final version submitted.

Author Disclosure

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