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Research Report

The potential of chitosan combined with chicken shank collagen as scaffold on bone defect regeneration process in *Rattus norvegicus*

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ABSTRACT

Background: *In the field of dentistry, alveolar bone damage can be caused by periodontal disease, traumatic injury due to tooth extraction, cyst enucleation, and tumor surgery. One of the ways to regenerate the bone defect is using graft scaffold. Thus, combination of chitosan and collagen can stimulate osteogenesis.* **Purpose:** *The aim of this study was to examine the potential of chitosan combined with chicken shank collagen on bone defect regeneration process.* **Method:** *Twelve Rattus norvegicus were prepared as animal models in this research. A bone defect was intentionally created at both of the right and left femoral bones of the models. Next, 24 samples were divided into four groups, namely Group 1 using chitosan – collagen scaffold (50:50), Group 2 using chitosan collagen-scaffold (80:20), Group 3 using chitosan scaffold only, and Control Group using 3% CMC-Na. On 14th day, those animals were sacrificed, and histopathological anatomy examination was conducted to observe osteoclast cells. In addition, immunohistochemistry examination was also performed to observe RANKL expressions.* **Result:** *There was a significant difference in RANKL expressions among the groups, except between Group 3 using chitosan scaffold only and control group (p value > 0.05). The highest expression of RANKL was found in Group 1 with chitosan – collagen scaffold (50:50), followed by Group 2 with chitosan-collagen scaffold (80:20). Moreover, there was also a significant difference in osteoclast generation, except between Group 1 using chitosan – collagen scaffold (50:50) and Group 2 using chitosan-collagen scaffold (80:20), p value < 0.05; and between Group 3 using chitosan scaffold only and control group, p value > 0.05. Less osteoclast was found in the groups using chitosan – collagen scaffold (Group 1 and Group 2).* **Conclusion:** *Combination of chitosan and chicken shank collagen scaffold can improve regeneration process of bone defect in Rattus novergicus animals through increasing of RANKL expressions, and decreasing of osteoclast.*

Keywords: *chitosan scaffold; chicken shank collagen; bone regeneration*

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INTRODUCTION

In the field of dentistry, alveolar bone damage can be caused by periodontal disease, traumatic injury due to tooth extraction, post-cyst enucleation, and post-tumor surgery. Biotechnology development actually has already introduced tissue engineering concept by using xenografts to help bone regeneration process since it does not require additional surgery, cause low morbidity, and reduce the risk of disease transmission.[2] However, in the last few years, innovation of bone tissue engineering has been developed, as a result, biomaterials are focused on a physicochemically suitable scaffold design for cell attachment, proliferation, differentiation, and specific organ tissue formation.[3]

Scaffold is the term for the synthesis of the extracellular matrix. Scaffold also becomes a place of attachment and growth of new cells. Thus, scaffold must be made of biodegradable materials that can be metabolized in the

body and eventually disappear when the new cells have started to grow a lot, be healthy, and survive. Some of the polymeric materials, which have been developed in tissue engineering, are chitosan and collagen.[4]

Chitosan is an amino polysacharide (poly-1,4-D-glucosamine), widely used as polymers in tissue engineering.[5] These polymers have been regarded as a material which has many functional advantages because it has high biocompatibility, biodegradable properties, and low toxicity.[6,7] Chitosan is also known to stimulate the growth and differentiation of osteoblasts in cell culture. The existence of chitosan alone is not osteoconductive enough, so the ability in new bone formation is still less than optimal. Approaches to overcome the weaknesses has been conducted, such as designing a composite by combining the strengths of different materials to minimize the weaknesses of two different materials.[8]

Another organic material playing an important role in tissue engineering is collagen.[9] Collagen can be found in cartilage, bone, intervertebral disc, blood vessels, tendons, ligaments, skin, and a major component of the extracellular matrix. Collagen can also be found in chicken shank. 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 cells.[10]

Collagen, furthermore, has been used in numerous applications in tissue engineering since it has good biocompatibility and biodegradable properties, as well as low antigenicity.[11,12] This material, thus, is considered as a material suitable for repairing damaged tissue and organ.[9] Collagen actually has many disadvantages, such as rapid degradation time and weak mechanical strength. Therefore, a combination of the two polymer materials is needed to produce better material. Chitosan and collagen can be combined into a new material to form a unique structure that can improve the mechanical strength and decrease the biodegradation rate of collagenase.[13,14] The concentrations used to make a good combination of chitosan and collagen scaffold with good mechanical strength are 50: 50[15,16] and 80: 20.[17]

In addition, bone healing is characterized by a series of cellular and molecular processes, as well as tissue transformation consisted of resorption and formation of hard and soft tissues. Bone formation by osteoblasts and bone resorption by osteoclasts regulate skeletal remodeling.[18] These processes are fundamental in maintaining bone mass and architecture.[19] Osteoclasts play an important role in bone resorption both physiologically and pathologically.

Osteoclasts require the presence of cytokine receptor activator of nuclear factor-κB ligand (RANKL) as a key cytokine inducing osteoclastogenesis.[20] In this research, two organic natural ingredients were used, namely chitosan combined with chicken shank collagen as a scaffold to determine the potential of those material on the healing process of bone defects in Rattus norvegicus rats on day 14 with by observing RANKL expressions and

osteoclast count. Similarly, a previous research also show that bone callus would increase on the 14th day after the administration of bone defect.[21]

MATERIALS AND METHODS

This research was an experimental laboratory research. Extraction process of chicken shank collagen and manufacture of chitosan and collagen scaffold were performed in Unit Research Services (ULP) of Pharmacy Faculty, Universitas Airlangga and the 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. Histological and immunohistochemical preparations was carried out in Diagnostic Center - Dr. Soetomo Hospital in Surabaya.

Gel base for the control group was made of carboxy methyl celulose sodium (CMC Na) at a concentration of 3%. Chitosan used in this research was chitosan with the degree of deacetylation of >75-85%. Chitosan gel was made by 200 mg of chitosan powder mixed with 5 ml of 0.1M acetic acid, and then added with 15 ml of 0.1 M NaOH. After that, centrifuge at 9000 rpm was carried out to obtain pure chitosan gel.

Moreover, collagen was obtained from the extraction of chicken shank collagen. Chicken shank was cut into small pieces and mashed. Smoothed chicken shank was mixed with 250 U/ mg of trypsin enzyme powder, and then incubated at 37° C for 24 hours. Glacial acetic acid was added and stored at 4° C for 48 hours. The preparations were mixed using a mixer to form a fiber, and then centrifuged to obtain a supernatant. They were centrifuged twice to obtain a pure supernatant. Supernatant obtained was mixed with 0.5M acetic acid to dissolve, and then added with 5% NaCl to form bands of collagen. The process was repeated three times. Those collagen bands were filtered with filter paper. After that, the collagen was dialysed and centrifuged again.

Furthermore, manufacture of chitosan and collagen scaffold was conducted by mixing chitosan gel and chicken shank collagen gel homogeneously with ratios of 50:50 and 80:20. The gel was inserted into scaffold mold made of teflon, then frozen at -20° C for 2 hours, and was followed by freeze dry for 24 hours. Scaffold then was sterilized using a clean bench UV.
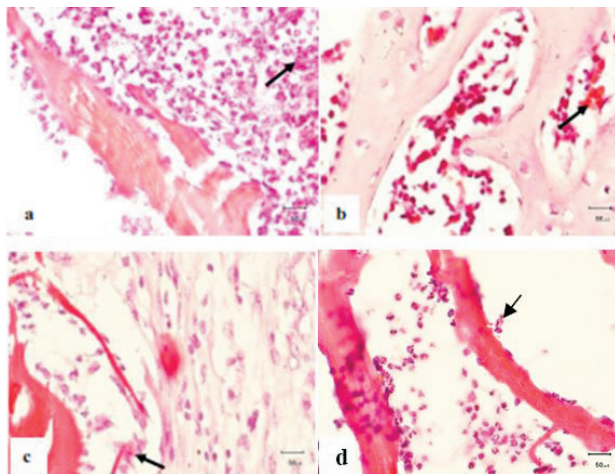
In addition, samples used were 12 male Wistar rats (Rattus norvegicus) weighed 250-300 mg. Their left and right femur bone then was defected. Thus, the total of samples used in this research was 24 samples. Those samples were classified into four groups each of which consisted of six samples. The research groups were control group (3% CMC Na), Group 1 using chitosan – collagen scaffold (50:50), Group 2 using chitosan collagen-scaffold (80:20), and Group 3 using chitosan scaffold only. Femur bone defect then was made with a diameter and height of

3 mm using a low-speed round bur under anesthesia condition using ketamine and xylazine. The scaffold material then was applied to the defected bone in accordance with the treatment of each group.

On day 14, the defected bones in the control and treatment groups were taken under the effects of inhalation anesthetics. Those bones were processed for the manufacture of histological preparations with hematoxylin eosin staining (HE) to examine the number of osteoclasts. Immunohistochemical examination then was carried out using RANKL antibody to observe RANKL expressions. Finally, osteoclast count and RANKL expressions were measured manually on five of the visual field examination using a light microscope with a magnification of 400x.

RESULTS

The results of this research showed the average values of osteoclast count and RANKL expressions in the control group, the treatment group using chitosan scaffold only, the treatment group using chitosan-collagen scaffold with the ratio of 80:20, and the treatment group using chitosan-collagen scaffold with the ratio of 50:50 during the healing process of those femur bone defect as shown in Table 1.

The most RANKL expressed by osteoblasts was found in the group using chitosan-collagen scaffold with the ratio of 50:50. Meanwhile, the least RANKL expressed by osteoblasts was in the control group. The highest number of osteoclasts was widely obtained in the control group, while the least number of osteoclasts was in the group using chitosan-collagen scaffold with the ratio of 50:50. The results of histopathological examination then were obtained by observing the number of osteoclasts in the control and treatment groups using a light microscope with a magnification of 400x as seen in Figure 1.

The fewest osteoclasts were found in the group using of chitosan-collagen scaffold with the ratio of 50:50 (d), following the group using of chitosan-collagen scaffold with the ratio of 80:20 (c), the group using chitosan (b), and the control group (a) (Figure 1). RANKL expressions in each treatment group and the control can be seen in Figure 2.

The illustration photo above show the results of immunohistochemical examination that the combination of chitosan and chicken shank collagen scaffold can increase RANKL expressions during the healing process of the fermur bone defect. However, the combination of chitosan-collagen scaffold with the ratio of 50:50 was more effective in increasing RANKL expression than with the ratio of 80:20.
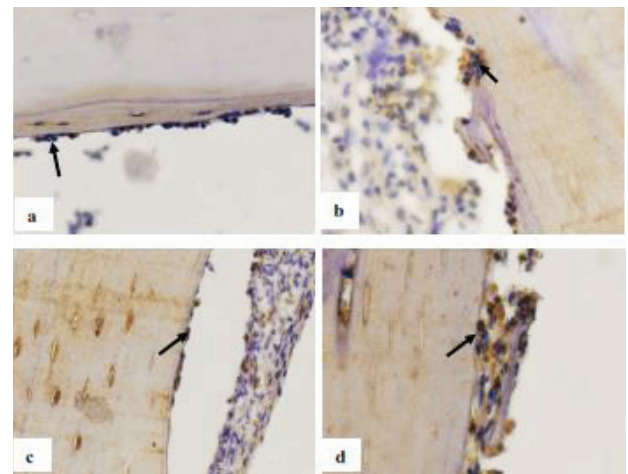

Figure 1. Osteoclast cells in each group. a) the control group; b) the group using chitosan scaffold only; c) the group using chitosan collagen-scaffold (80:20); d) the group using chitosan–collagen scaffold (50:50).

Figure 2. RANKL expressions in each group. a) the control group; b) the group using chitosan scaffold only; c) the group using chitosan collagen-scaffold (80:20); d) the group using chitosan – collagen scaffold (50:50).

Table 1. The average values of osteoclast count and RANKL expressions

Group	Number of samples	Average values (Mean)	
		Osteoclast count	RANKL expressions
Control	6	4.8333	5.8333
Chitosan scaffold	6	4.6667	8.1667
Chitosan scaffold with the ratio of 80:20	6	2.1667	17.5000
Chitosan scaffold with the ratio of 50:50	6	1.3333	28.0000

The research data of osteoclast count and RANKL expressions in each group were then tested using one way Anova test. Before the one way Anova test conducted, the results of Kolmogorov Smirnov test showed that the distribution of the data in this research was normal (p> 0.05). Based on one way Anova test, the significance value obtained was 0.000 (p<0.05). It means that there was a significant difference in osteoclast count and RANKL expressions between the control group, the group using chitosan scaffold only, the group using chitosan collagen-scaffold (80:20), and the group using chitosan–collagen scaffold (50:50). Thus, Post Hoc test and Tukey HSD test were performed to know differences between one group and another group.

There was a significant difference in the number of osteoclast cells between one group and another group. Nevertheless, there was no significant difference in the number of osteoclast cells (p>0.05) between the group using chitosan scaffold only and the control group, as well as between the group using chitosan-collagen scaffold with the ratio of 50:50 and the group using chitosan-collagen scaffold with the ratio of 80:20. In addition, there were significant differences in RANKL expressions between one group and another group. However, there was no significant difference in RANKL expressions (p>0.05) between the group using chitosan scaffold and the control group (p> 0.05).

DISCUSSION

On the 14th day, histopathological anatomy observation was conducted to examine the number of osteoclasts. Osteoclasts play an important role in bone resorption, both in physiological and pathological conditions. Osteoclast are derived from myeloid cells and macrophages, producing many cytokines and regulating macrophages and dendritic function. Osteoclast, moreover, are located on the surface of endosteal cells in the Havers channels along cortical and trabecular bones.[22] Osteoclasts are the largest, multinucleated, irregularly shaped cells with pale cytoplasm color.[23] The least average number of osteoclasts was found in the group using chitosan – collagen scaffold (50:50), following the group using chitosan–collagen scaffold (80:20), and the group using chitosan scaffold only. The highest average number of osteoclasts was found in the control group.

Osteoclasts, furthermore, are known to contribute to bone resorption. As a result, the least number of osteoclasts was found in the groups using chitosan–collagen scaffold. It may indicate that bone formation is stimulated dominantly by osteoblasts during the healing process of the femur bone defect in those experimental animals. RGD (Arg-Gly-Asp) contained in the collagen can inhibit the expression of RANK by blocking the integrin, $\alpha v \beta 3$, so possibility of RANKL to bind to RANK is lower and inhibits the formation of mature osteoclasts.[24]

The statistical results, however, showed that there were significant differences between each groups, except between the group using chitosan-collagen scaffold with the ratio of 50:50 and the group using chitosan-collagen scaffold with the ratio of 80:20, as well as between the group using chitosan scaffold only and the control group (p>0.05). This is due to the fact that there was not much different in the average pore size of the scaffold between in the the group using chitosan-collagen scaffold with the ratio of 50:50 and in the group using chitosan-collagen scaffold with the ratio of 80:20 about 183 m and 123 m. Thus, there was no significant effect in reducing the number of osteoclasts useful in the process of bone healing. The greater porosity size actually can trigger a better vascularization so that the healing process can be more optimal.[17] Both the treatment group using chitosan scaffold only and the control group, consequently, did not produce statistically significant differences in the number of osteoclasts because the chitosan alone is not enough osteoconductive. Therefore, the bone healing process was less than optimal. In this case, the ability to produce osteoclasts was not much different between the control group and the treatment group using 3% CMC-Na on the 14th day.

The expressions of RANKL by osteoblasts, moreover, were more visible in the treatment groups using chitosan-collagen scaffold combination than the group using chitosan scaffold only and the control group due to a possible increase in the number of osteoblasts and the expression of osteoprotegerin (OPG), playing important roles in bone formation. In other words, the combination of chitosan-collagen scaffold can stimulate the occurrence of osteogenesis by facilitating adhesion, proliferation, and differentiation of cells. Collagen can also enhance osteoblast differentiation and increase bone formation by activating genes RUNX-2 to stimulate pre-osteoblasts into osteoblas.[25] Therefore, it can be said that the greater level OPG expressions than the level of RANKL expression may trigger bone formation. RANKL is a key cytokine in stimulating osteoclastogenesis.[19] RANKL can stimulate differentiation, maintain viability, and activate mature osteoclasts.

All those functions can be run because of the interaction between RANKL and receptor activator of nuclear factor-κB (RANK). RANK is a transmembrane protein expressed by the pre-osteoclasts. In the bone healing process, there is a protein, OPG, which can inhibit osteoclast development. OPG functions as a decoy receptor by binding to RANKL, resulting in inhibiting RANK signaling.[26] Besides, since RANKL is expressed by osteoblasts, the high number of osteoblasts may also contribute to the high expressions of RANKL. According to a research conducted by Wang, RANKL and OPG levels can significantly increase immediately after a fracture in a bone fracture until the 4th week compared to the control group of healthy bone.[27] Although both increase, the presence of RANKL is less than OPG, thus showing that the number of osteoblasts will increase over that period and bone formation is more

dominant in playing the role during the bone healing process.

Finally, the results of this research need to be analyzed further. Further researches should reveal the location of RANK and OPG expressions since RANK/ RANKL/ OPG is a signaling system that is responsive to control osteoclastogenesis. Properties of chitosan itself have good mechanical strength less than in combination with other polymers so that chitosan scaffold is less optimal in facilitating osteogenesis. In this case, the ability to express RANKL in bone defects was not much different from the control group on the 14th day. It can be concluded that the combination of chitosan and collagen scaffold can improve the healing process of bone defect in Wistar rats through an increase in RANKL expressions and a decrease in the number of osteoclasts.

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