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Research Report

Scaffold combination of chitosan and collagen synthesized from chicken feet induces osteoblast and osteoprotegerin expression in bone healing process of mice

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ABSTRACT

Background: *Over 500.000 of the 2,3 million surgical treatments requiring bone grafting procedures that are performed annually are likely to be necessitated by or will result in bone defects that will not regenerate. Treatment to regenerate new tissues is needed, especially for hard tissue repair, which not only relies on a natural osseointegration process, but also requires a physical support to guide the differentiation and proliferation of cells into the targeted functional tissue. Chitosan and collagen extracted from chicken feet combinations are expected to enhance the bioactive surface and provide mechanical strength as a bone graft scaffold.* **Purpose:** *The aim of this study was to investigate the role of chitosan and collagen scaffold synthesized from chicken feet applications to increase the expression of Osteoprotegerin (OPG) and osteoblast cells on the fourteenth day of bone healing.* **Methods:** *Eighteen three-month old, adult, male, Rattus novergicu strain rodents with a body weight ranging from 200-350 g were kept under controlled environmental conditions. The mice were randomly divided into three groups consisting of three subjects, each treated with collagen, chitosan, chitosan-collagen combination (50:50) scaffolds. On the 14th post-treatment day, three members of each group were sacrificed. Examination of Osteoprotegerin (OPG) expression was conducted by means of immunohistochemistry staining with anti-OPG polyclonal antibodies. Meanwhile, osteoblast cell examination was performed by means of hematoxilin-eosin (HE) staining.* **Results:** *The mice treated with collagen and a chitosan-collagen combination scaffold presented an increase in the expression of Osteoprotegerin (OPG) and the number of osteoblast cells respectively.* **Conclusion:** *A combination of chitosan-collagen (50:50) scaffold extracted from chicken feet increased the expression of OPG and the number of osteoblasts in the bone healing process. The combination scaffolds demonstrated the highest OPG expression and number of osteoblasts compared to the other groups.*

Keywords: *collagen; chitosan; scaffold; chicken feet; bone healing*

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INTRODUCTION

Debridement is a surgical procedure resulting in massive tissue loss. More than 2,300,000 operations have been recorded and over 500,000 bone replacements involving the use of grafts are performed annually as forms of health care.[1–3] Thus, a therapy to regenerate new tissues is required. Treatments for tissue and bone defects incorporating tissue engineering methods, such as the use of bone graft and stem cells, have been developed as an alternative to conventional defect treatments.[4]

In recent decades, treatments involving the use of grafts have represented a novel approach to tissue and bone repair. Tissue engineering methods primarily intended for hard tissue repair not only rely on natural osteointegrative processes, but also on a material promoting osteointegration which is the bone graft.[5] In bone tissue engineering, a bone graft is formed into a scaffold for attachment, proliferation

and differentiation of bone tissue cells,[6,7] to replace, repair and regenerate damaged tissue.[8] Currently, there are three kinds of natural bone graft widely used in the medical field, namely; autograft, a bone substitute derived from the patient him/herself, allograft, bone substitute provided by human donors and xenografts and bone substitutes derived from other species, such as cows. Autograft has several disadvantages: the need for surgery to remove bones from the donor potentially resulting in clinical problems, the limited availability of bones and the risk of death. Certain allograft materials and xenograft have the drawback of possibly inducing autoimmune reactions, while the nature of the osteoinduction of materials is less than optimal.[9,10]

Chitosan constitutes a natural polymer alloplast and bone replacement material whose use in biomedical field applications has attracted considerable attention due to its biodegradability, biocompatibility, antibacterial and regenerative properties, all of which can accelerate tissue and bone healing.[11] Poly[-(1,4)-2-amino-2-deoxy-D-glucopiranose] or chitosan is a natural biopoly-aminoaccharide obtained from the stable deacetylation of chitin. However, the use of chitosan alone in tissue regeneration is less than optimal because it is incapable of entirely replacing the bone tissue.[12]

In addition to chitosan, another biomaterial renowned as a tissue substitute is collagen which constitutes a group of proteins with special characteristics, found in all multicellular animals, and secreted by connective tissue and various other cells. The synthesis of collagen was originally thought to be confined to fibroblasts, condroblasts, osteoblasts and odontoblasts. However, it later turned out that this material can be synthesized by various cells. Most collagen is synthesized in fibroblasts, whereas bone collagen is produced by osteoblasts and cartilage collagen by condroblasts respectively. In experimental studies, collagen has been shown to reconstruct damaged tissue and, being one of the main components of bone, offers hope for positive tissue reaction.[11,13] In this study, the synthesis of collagen scaffold from chicken feet was combined with chitosan in an attempt to analyze and identify the potential role of collagen combination scaffolding of chicken feet and chitosan in accelerating the bone healing process in mice.

MATERIALS AND METHODS

This research was accepted by the Ethics Committee of the Faculty of Dental Medicine of Universitas Airlangga, No. 45/KKEPK.FKG/IV/2015. It represented an experimental in vivo laboratory research with post test-only control group design. Three treatment groups were established, each treated with collagen, chitosan and chitosan-collagen (50:50) scaffolds.

The research subjects were randomized and divided into three groups, namely; the collagen, chitosan and chitosan-collagen scaffold treatment groups respectively. They were subsequently adapted to the environment over seven days, with all receiving basal rations. Basal ration composition, consisting of carbohydrates, proteins, fats, minerals, vitamins and water, was prepared according to American Institute of Nutrition (AIN) standards.[14]

The collagen was synthesized from a broiler of chicken feet skins obtained from PT. Wonokoyo. The chicken feet were cut into small pieces, mixed with trypsin enzyme and placed in an incubator at a temperature of 37^0C for 24 hours. This mixture was added to glacial acetic acid and then agitated with a mixer until the formation of fiber was observed. The synthesized results were centrifuged at 9000 rpm with the supernatant being extracted to obtain collagen. The supernatant was subsequently added together with 5% NaCl to the formation of fibers/collagen bands. The extraction by means of acetic acid and sodium hydroxide was analyzed using a cellophane membrane (Sigma, 58188). The results of dialysis can be formed using a mold/scaffold mold and then freeze dried.

The preparation of a combination of chitosan collagen scaffold was based on a weight ratio of 50:50. The chitosan (Sigma, SMB00279) gel was obtained from Sigma brand chitosan powder at 85% deacetylation that had been dissolved with an acid base and then added to collagen gel and acetic acid. The chitosan and collagen gel mixture was agitated and centrifuged at 9000 rpm. The resulting supernatant was subsequently inserted into the mould scaffold, enabling it to be frozen for 24 hours.

Prior to surgery, the three month-old, male rats were anesthetized. Bone defects in two areas of smelting (one on the right and the other on the left) of 5 mm were produced using Round Burs Angle (Dentsply, 63503001) on their femur bones. After these defects had been made, they were administered the collagen scaffold, chitosan scaffold and 50:50 chitosan-collagen scaffold. Thereafter, a suture was performed on the wound with 3/0 non-absorbable black silk (Sinorgmed, China). On the 14th post-operative day, members of each group was sacrificed to enable observation of the degree of osteoblast cell and OPG expression as an indicator of bone regeneration.

The femoral bone tissue taken from the animal was tested with 10% formalin buffer solution before being decalcated by means of 2% nitric acid. The tissue processing continued involving dehydration, clearing, impregnation, embedding, tissue cutting and coloring. Morphology and the number of osteoblast cells were investigated using a light microsope, while staining by means of hematoxylin-eosin was conducted. In order to observe the expression of osteoprotegerin, immunohistochemical imaging using anti-OPG (Bioss, bs-0431R) polyclonal antibodies was conducted. The data of this study were subsequently analyzed through the application of one-way ANOVA and Tukey HSD tests.

RESULTS

On cellular examination involving hematoxilyn-eosin staining, the visible osteoblast cells were found to be single-core hexagonal-shaped cells often present at the edges of the bone matrix. Inspection was carried out with a light microscope at 400x magnification. The results of the examination conducted on the fourteenth day can be seen in Figure 1. The results show that a combination treatment involving collagen or chitosan scaffolds results in a more pronounced increase in osteoblasts than treatment without combination.

From the results of the Kolmogorov-Smirnov Test statistical analysis of data, the p value (2-tailed) amounted to 0.296> 0.05. Thus, it could be argued that the data was normally distributed. A homogeneity test was subsequently administered by means of a Lavene test which produced a p value of 0.15> 0.05, indicating that the data was homogeneous or demonstrated the same variance. Therefore, the data was valid for the parametric test using one-way ANOVA. From the results, it could be seen that the p value was 0.000 <0.05 meaning that there was a significant difference between treatments. Consequently, a post-hoc Tukey HSD test was administered which showed that chitosan scaffold treatment was not significantly different (*p value* 0.38>0.05) to collagen scaffold treatment, but chitosan scaffold was significantly different compared to 50:50 chitosan-collagen scaffold combination.

Immunohistochemistry examination incorporating the use of a polyclonal antibody against OPG was conducted. Positive results were characterized by the presence of brown spots on the cytoplasm of osteoblasts. Checked with a light microscope at 400x magnification, the results of the 14th day observation and examination can be seen in Figure 2. The arrows indicate a positive result as confirmed by the brownish color on the osteoblast cell cytoplasm. The results show that combination treatment produces increased osteoprotegerin expression in osteoblasts compared with non-combination treatment, i.e collagen or chitosan scaffolds.

From the statistical analysis of the data, the Kolmogorov-Smirnov Test recorded a p (2-tailed) value of 0.350> 0.05. Hence, it can be said that the data was normally distributed. A homogenity test using Lavene's test produced the p value of 0.20> 0.05 which means that the data was homogeneous or presented the same variance. Therefore, the data was valid for the parametric test using one-way ANOVA. The results of several such tests using one-way ANOVA confirmed the p value as 0.000 <0.05 which means that there was a considerable difference between treatments. The subsequent post-hoc Tukey HSD confirmed that, while chitosan scaffold treatment was not significantly different (*p* value 0.06>0.05) from a collagen scaffold treatment, 50:50 chitosan-collagen combination scaffolds contrasted sharply with chitosan scaffold.

DISCUSSION

The regeneration of bone tissue requires an artificial structure, or so-called scaffold, as a location for tissue growth that maintains tissue mechanical stability, thereby
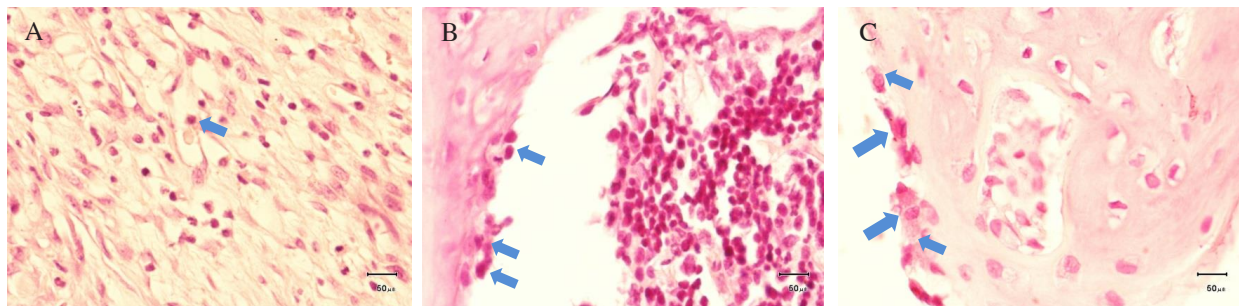

Figure 1. Image of osteoblast cell featuring the Hematoxylin-Eosin staining at 400x magnification under treatment a. chitosan, b. collagen, c. chitosan-collagen 50:50 on the 14th day of observation.
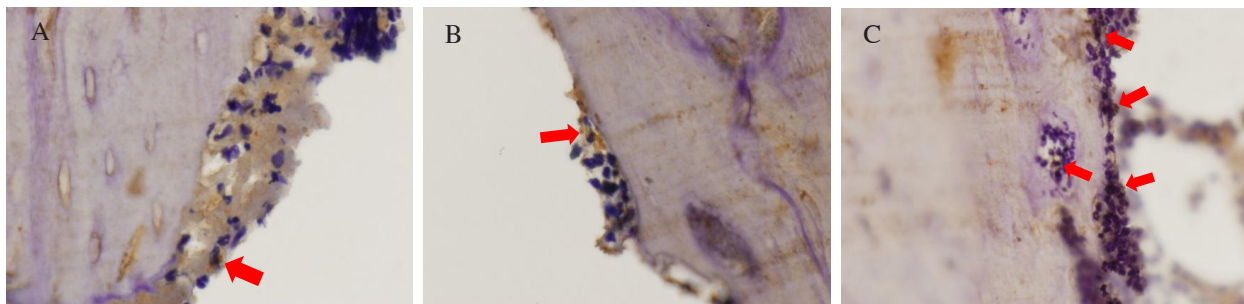

Figure 2. Brownish images of the osteoblast cell cytoplasms of imunohistochemical imaging that show OPG (400 magnification) in treatment a. chitosan, b. collagen, c. chitosan-collagen 50:50, on the 14th day.

allowing bone defects to be restored to their original form.[1] Collagen is considered to be the most promising material for tissue engineering applications because of its excellent biocompatibility, degradability, low antigenicity and abundance in mammals. Like collagen, chitosan has been utilised in a variety of biomedical fields. including skin tissue engineering. In addition to being antibacterial, Chitosan has specific properties including; bioactivity, biocompatibility, and biodegradability. The quality of chitosan can be seen from its intrinsic properties, its purity, molecular mass, and deacetylation degree of 75-100%. The degree of deacetylation of chitosan affects the physico-chemical properties of polysaccharides, such as the rheological nature of chitosan and the flexibility of the molecular chains. The ideal scaffold would consist of a biodegradable material possessing a pore structure that can provide a microenvironment for osteogenesis and osteoblast cell proliferation. Scaffolds made from chitosan have been widely used as a biomedical material because of their non-toxicity and osteoconductivity. Scaffolds made from collagen represent the most suitable material to repair damaged tissue because it is the main protein structure in bone.

In this study, the average number of osteoblasts and OPG in collagen and chitosan scaffolds was lower than in the 50:50 chitosan-collagen combination group. Chitosan-collagen combinations in the form of scaffolds are normally used for attaching and cell migration, delivering and maintaining the cells from biochemical factors, enabling the diffusion of vital cell nutrients and both producing and exerting mechanical and certain biologic influence in order to modify the behavior of the cell phase.[15,16] In other studies revealed that osteoblasts increased significantly at the outset of the 20 days of addition of the scaffold in the experimental specimens,[17] however, in our study blood vessels were formed on Day 14,[18,19] and osteogenesis started on the same day after the graft was implanted in the bone, so the observation of this study was on day 14.[15]

50:50 chitosan-collagen scaffold treatment is significantly different to collagen and chitosan scaffolds because the latter are porous ± 650μm-850μm.[20] The

pore size is too large for the scaffold whose mechanical properties it can influence. These properties are essential for tissue repair as they affect the function of certain tissue cells, as well as attachment, migration and cell proliferation in tissues.[1,21] Therefore, multiple or combination agents are rapidly provided to the wound through the normal bone healing process. The collagen treatment provides protein in the form of a matrix in which cells can proliferate and infiltrate. In addition to providing the cells with a matrix largely lost during wound creation, the collagen scaffold was observed to activate platelets within the chitosan combination. The greater the diameter of the pores, the less the extent to which mechanical stability of the tissue is maintained resulting in the healing process being the same as in the group without the addition of the scaffold.[22,23] Collagen and chitosan are good natural ingredients used as tissue engineering materials but when used separately they inhibit the growth of new blood vessels transporting new bone nutrients and decrease the mechanical properties of the scaffold. The chitosan-collagen combination scaffold is more stable because the chitosan content of the combination scaffold can serve as a bridge that increases the efficiency of the bonds between the amino acid of the chitosan-collagen chains in the tissue.[24–26] When used separately, a scaffold of chitosan and collagen is less conducive to bone healing because one ingredient is too rapidly degraded by the body. Therefore, the scaffold that serves as a cell infiltration site and guide for the differentiation and proliferation of cells into functional tissue does not function optimally.[24] Strong bonds between amino acid chains within the combination of chitosan-collagen scaffold cannot easily be degraded in the tissues, thus increasing the latters' mechanical strength and structure.[27] It can be concluded that therapy incorporating the application of a chitosan-collagen scaffold combination derived from chicken feet can increase the number of osteoblast cells and OPG expression in the healing process of bone defects in mice.

Figure 3. Average osteoblast cell count and osteoprotegerin expression on day 14

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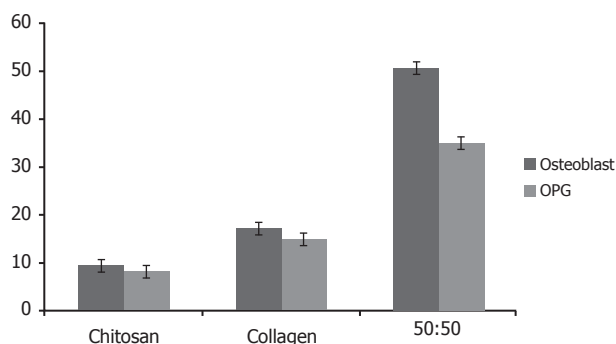6. Lanza RP, Langer R, Vacanti J. Principles of Tissue Engineering. 2nd ed. Academic Press; 2000. p. 995.

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