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Research Report

Application of chitosan scaffolds on vascular endothelial growth factor and fibroblast growth factor 2 expressions in tissue engineering principles

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ABSTRACT:

***Background**: Tissue engineering has given satisfactory results as biological tissue substitutes to restore, replace, or regenerate tissues that have a defect. Chitosan is an organic biomaterial often used in the biomedical field. Chitosan has biocompatible, antifungal, and antibacterial properties. Chitosan is osteoconductive, suitable for bone regeneration applications. Bone defect healing begins with inflammatory phase as a response to the presence of vascular injury, so new vascularization is required. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (FGF2) are indicators of the beginning of bone regeneration process, playing an important role in angiogenesis. **Purpose:** This research aims to determine the effects of chitosan scaffold application on the expressions of VEGF and FGF2 in tissue engineering principles. **Method:** Chitosan was dissolved in CH_3COOH and NaOH to form a gel. Chitosan gel was then printed in mould to freeze dry for 24 hours. Those rats with defected bones were divided into two groups. Group 1 was the control group which defected bones were not administrated with chitosan scaffolds. Group 2 was the treatment group which defected bones were administrated with chitosan scaffolds. Those rats were sacrificed on day 14. Tissue preparations were made, and then immunohistochemical staining was conducted. Finally, a statistical analysis was conducted using Kruskal test. **Result:** There was no significant difference in the expressions of VEGF and FGF2 between the control group and the treatment group ($p>0.05$). **Conclusion:** Chitosan scaffolds do not affect the expressions of VEGF and FGF2 during bone regeneration process on day 14 in tissue engineering principles*

***Keywords:** chitosan; VEGF; FGF2; tissue engineering*

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INTRODUCTION

Tissue engineering is an application of the principles and methods of engineering used to restore, maintain, or improve tissue function. The principles of tissue engineering are conducted by providing appropriate materials to trigger the cells in order to regenerate or combine the performance of the cells in the body by using *scaffold* to trigger the growth of new tissue. Tissue engineering has given satisfactory results as biological tissue substitutes to restore, replace, or regenerate tissue suffering from defects.[1] Cells, *scaffold,* and growth stimulating signals are generally called as tissue engineering *triad,* a major component of tissue engineering.

Scaffold is an important material in tissue engineering. *Scaffold* is a very nucleolus artificial extracellular matrix used for cell accommodation, cell growth, and tissue regeneration.[2] *Scaffold* must meet requirements. For instance, *Scaffold* must have interconnecting pores appropriate to support tissue integration and vascularity, made of materials that have certain properties, such as biodegradation or bioresorption. As a result, the tissue will eventually be replaced with *scaffolds*, which have a surface suitable for supporting cell attachment, cell differentiation, and cell proliferation, as well as have good mechanical properties, easily made into a variety of shape and size.[3]

Organic biomaterials are often used in the biomedical field, especially chitosan application. Chitosan has biocompatibility, antifungi, and antibacteria properties.[4] Chitosan also is osteoconductive, so it is suitable for hard tissue regeneration application, but the mechanical properties and biological activities need to be improved.[5] The biological properties of chitosan, very influential in the process of wound healing process, are biodegradability, biocompatibility, and antimicroba. The high biodegradation rates can trigger chitosan to be increasingly inadequate in accelerating wound healing. Biodegradability of chitosan also affects high biocompatibility because degradation will increase amino sugar accumulation and inflammatory response.[6]

Bone healing process is a series of molecular and cellular process as well as tissue transformation, from resorption to hard and soft tissue formation. Bone defect healing process begins with inflammatory phase, which is vascular response to injury, requiring new vascularization. Vascularization plays an important role in osteogenesis during the bone healing process.[7-9] Increased formation of blood vessels in the area indicates that wound epithelialization process occurs faster.[10]

Bone regeneration can be affected by vascular endothelial growth factor (VEGF) directly or indirectly. VEGF plays an important role in the formation of new blood vessels serving to mobilize and recruit endothelial progenitor cell (EPC), a well as to differentiate and proliferate endothelial cells.[8] VEGF induces angiogenic process through endothelial cells. Bone-forming precursor cells migrate through the bloodstream to the callus that will differentiate into osteoblasts.[9] VEGF affects osteogenesis from day 14 to day 21 after the defect occurred.

In addition to VEGF, basic fibroblast growth factor (FGF2) plays an important role in the process of vascularization. VEGF and FGF2 can also stimulate fibroblasts to migrate to the defect and trigger collagen synthesis. FGF2 expression occurs in the early phase of bone healing process to form osteoblasts.[11] FGF2 also plays a role in mitogenesis of mesenchymal cells, proliferating and differentiating into progenitor cells. Progenitor cells will differentiate into osteoblasts as bone formation cells.[13]

Finally, this research aims to determine the effects of chitosan scaffold application on VEGF and FGF2 expressions in tissue engineering principles. This research can be used as biomaterials in dentistry that are able to act as the basis of modern therapies based on tissue engineering.

MATERIALS AND METHOD

This research was a laboratory experimental research with post control group design. Animals used were male rats (*Rattus norvegicus*) aged 3 months old and weighed 250 grams. Materials used in this research were chitosan with deacetylation 81% (Sigma 93646, USA), a solution of acetic acid (CH3COOH) and NaOH (Merck, Germany), VEGF polyclonal antibody (BIOSSUSA), and FGF2 polyclonal antibody (BIOSSUSA). Tools used in this research were a glass beaker, connicle tube, freezer (Royal Chest Freezer BD 195, China), spatula glass, scales (Pioneer, USA), magnetic stirrer (HANNA, USA), and freeze-dryer (Heto FD3, EN 87 164, Japan).

Chitosan *scaffold* was synthesized by dissolving chitosan powder into a solution of acetic acid (CH3COOH 0.5M) and sodium hydroxide (NaOH 0.1M), and then centrifuged at a speed of 9000 rpm for 10 minutes. Supernatant in the form of chitosan gel was inserted into the mold/mould. The mold already containing chitosan gel was frozen at a temperature of -20° C using deep freezer for 2 hours, and then freeze-drying was conducted for 24 hours to form a porous three-dimensional structure, known as scaffold.

Research procedure, furthermore, was performed into several phases. Those six rats were divided into two groups. Group 1 was the control group, while Group 2 was the treatment group. Those rats were acclimatized for one week. The manufacture of bone defects was performed in both groups by drilling the rats' femoral and dextral areas. During the making of bone defects, irrigation was made using Ringer solution. In the defect area of the control group, placebo scaffold was applied to each defect using tweezers and escavator, and then sewed with 3/0 non-absorbable black-silk thread on muscles. In the defect area of the treatment group, chitosan scaffold was applied to each defect using tweezers and escavator, and then sewed with 3/0 non-absorbable black-silk thread on muscles.

On the 14th day after the closure of the defect, those rats were anesthetized using 10% ether as asphyxiation, and then sacrificed. The defected tissues of those rats were cut. The pieces of the tissues were fixed with 10% formalin for two days. They were decalcified using EDTA liquid up in order to soften the tissues to facilitate the process of cutting. The tissues were cut to a thickness of 0.3 mm. Dehydration process was carried out using alcohol. Those pieces of the tissues were put in xylol solution three times. Paraffin infiltration was conducted by melting solid paraffin. Embedding tissue was performed by pouring paraffin into molding devices. The tissues were taken using tweezers, and then implanted into the mould, which had been filled by paraffin. The mould, which had been filled by paraffin and tissue, was cooled to form paraffin blocks. Those rats that had been sacrificed were then buried properly.

Next, making immunohistochemical preparations were performed by cutting the paraffin block using a rotary microtome. The pieces placed in the glass object was then deparaffinized using xylol solution. The preparations were dripped with normal serum evenly on a glass object, and then put in a preparation box (humidity chamber) with tissue paper, etched with PBS to keep the moisture. Afterward, the preparations were put in an incubator at a temperature of 37° C for 45 minutes. Next, the tissues were dripped with primary antibody against inducible nitric oxyde synthase (iNOS) enzyme, and incubated at 4° C for one night. The tissues then were dripped with secondary antibody (biotinylation), and incubated at 37° C for 35 minutes. The administration of chromogen was performed by dripping a solution of 3,3'- diaminobenzidine (DAB), then incubated at 37° C for 35 minutes. Counter stain was conducted by dripping hematoxylin shed evenly, then left for 15 seconds and washed with running water. Next, dehydration was carried out with alcohol solution 70%, 80%, 90%, and

100%. Clearing with xylol (I, II, and III) and closing preparations (mounting) then were carried out immediately using the cover glass.

Finally, expressions of VEGF and FGF2 in each sample were assessed semiquantitatively using Remmele method that had been modified. Remmele scale index (immuno reactive score/IRS) is the result of multiplying immunereactive cells percentage score to color intensity score on the immunoreactive cells. Data then were obtained from the average value of the IRS in each sample observed in five different fields of view at 400x magnification. The whole of this examination was performed using a H600L brand Nikon light microscope equipped with a 300 megapixel DS Fi2 digital camera and image processing software, Nikkon Image System.

RESULTS

Based on the results of the statistical Kruskal Wallis test, p value obtained was less than 0.05, which means that there was no significant difference in VEGF and FGF2 expressions between the control group and the treatment group.

DISCUSSION

Tissue engineering involves cells as a building block, scaffold as a template, and growth factor as a biochemical signal that indicates there has been a growth of tissue. The primary function of scaffold is as cell support, artificial extracellular matrix not only providing sufficient mechanical environment of cells, but also causing cell attachment, proliferation, differentiation, and metabolism signals.[4] Selection of biomaterials for scaffold design, thus, is essential to cell growth and proliferation in three-dimensional matrix.

Chitosan is a natural polysaccharide which is similar to glycosaminoglycans and has a good interaction with cell membrane.[6] Natural polysaccharides can stimulate the activity of growth factors, which can maintain cell phenotype in particular morphology and play an important role as scaffold component of soft tissue and hard tissue.[7]

Chitosan-containing N-acetyl-D-glucosamine can bind to receptors that recognize macrophages. Macrophages produce VEGF directly to stimulate endothelial cell proliferation.[9] In addition, chitosan may provide more amino groups for the attachment and proliferation of endothelial cells because of the affinity between cations of the ammonium group of chitosan and the anion surface of the endothelial cell membrane.[13]

In this research, chitosan with a degree of deacetylation of 81% was used. Deacetylation degree can increase the attachment and stimulate fibroblast proliferation. Deacetylation degree of chitosan can also indicate free amine groups presented in the structure of chitosan. Cationic amine chitosan group provides a suitable environment for cell attachment. Cell attachment, however, is not only influenced by acetylation degree, but also by molecular weight and porosities. Chitosan, furthermore, can increase the proliferation of fibroblasts indirectly through the formation of poly-electrolyte complex with serum as heparin.[13]

Moreover, the results of this research showed that there was no significant difference in VEGF expressions between the treatment group and the control group on day 14 during the process of bone regeneration. Chitosan could stimulate inflammatory cells and growth factors in the early phase of wound healing process. This is supported by a research conducted by Inan and Saraydin indicating that the expression of VEGF in post-administration of chitosan will decline on day 14.[14] Chitosan could also stimulate the formation of granulation tissue. At the same time, chitosan could stimulate both fibroblasts to proliferate and extracellular matrix formation.

Besides, the results of this research also showed that there was no significant difference in FGF2 expressions between the treatment group and the control group on day 14 during the process of bone regeneration. Nevertheless, the research conducted by Inan and Saraydin showed that the number of FGF2 expressions on the 14th day is lower than on the 3rd day.[14] This condition is possible because fibroblasts have produced collagen fiber on the 14th day. Consequently, young fibroblasts will grow into inactive matured fibroblasts, known as fibrosis. In this research, however, chitosan scaffold on the 14th day could still interact with the endothelial cells and fibroblasts although not optimal. Finally, it may be concluded that chitosan scaffold cannot affect VEGF and FGF2 expressions on the 14th day during bone regeneration process in tissue engineering principles.

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Table 1. The average and standard deviation of VEGF and FGF2 expressions in the control and treatment groups

Group	$\overline{X} \pm SD$	
	VEGF	FGF2
Control	10.2 ± 2.7301	8.3 ± 3.0746
Treatment	6.93 ± 3.8070	3.4 ± 1.4422

Figure 1. The average of VEGF and FGF2 expression.

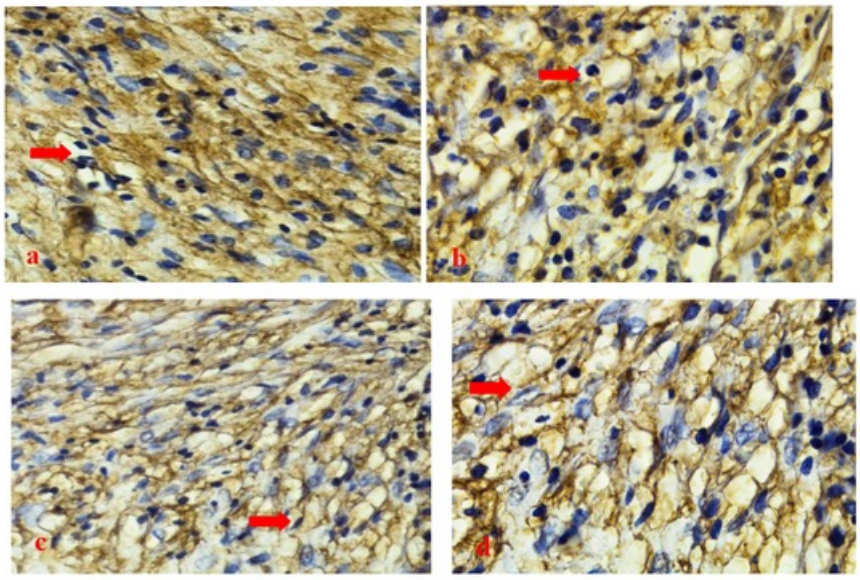

Figure 2. VEGF expressions on the control group (a) and the treatment group (b). FGF2 expressions on the control group (c) and the treatment group (d). VEGF and FGF2 expressions were evenly distributed in the control and treatment groups.

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