Vascular Endothelial Growth Factor Expression after Induced by Chicken Shank Collagen Scaffold in Bone Regeneration

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Abstract

Vascular endothelial growth factor (VEGF) is an essential mediator during the process of angiogenesis that is an initial process of bone regeneration. Engineered bone tissue commonly encompass the use of scaffold. Collagen plays an important role in bone regeneration. Unfortunately, collagen can be extracted from chicken shank.

This research was aimed to determine the effect of chicken shank collagen scaffold on the expresion of VEGF. Chicken shank was mixed by trypsin powder and dissolved in CH3COOH until forming like fiber then was sentifuged. Supernatant was dissolved NaCl 5wt% until forming fiber then was dialyzed for three days. Chicken shank collagen was printed in mould to freeze dry for 24 hours. Rats with defected femur were divided into 2 groups. Group 1 was the control group, and group 2 was treatment group which the defected femur were administrated with chicken shank collagen scaffold. Those rats were sacrificed on 2nd week. Tissue preparation was made then immunohistochemical staining was conducted counting VEGF expression. A statistical analysis was conducted using Mann Whitney test.

There was a significant difference in the expression of VEGF ($p<0,05$). The amount of VEGF expression was increased by chicken shank collagen scaffold.

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Introduction

A common approach in tissue engineering is to fabricate three-dimensional precursor tissue analogs from cells, scaffolds, and signaling molecules. Three-dimensional porousity known as scaffold plays an important role in manipulating cell function and guidance of new organ formation. Scaffold is a very nucleolus artificial extracellular matrix used for cell accommodation, cell growth, and tissue regeneration.[1]

An ideal scaffold for bone tissue engineering should have interconnected porous structure, good biocompatibility and good biodegradable.[2]

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Scaffold must have interconnecting pores appropriate to support tissue integration and vascularisation, made of materials that have certain properties, such as biodegradation. 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] The scaffold materials have a significant effect on cellular activity. They act as a physical support structure and as an insoluble regulator of biological activity that affects cell responses.

Collagen is a significant constituent of the natural extracellular matrix. Scaffolds made of collagen have been used in a variety of applications due to a number of useful properties such as hemostatic effect, low antigenicity, and good mechanical characteristics.[4] Collagen is known to be the most promising material for their excellent biocompatibility and biodegradability.[5] Due to its negligible immunogenicity, excellent

biocompatibility, mechanical stability and its ability to be involved in all 3 phases of the wound-healing cascade, studies have examined the use of collagen for wound dressing.[6]

Collagen possesses many abilities such as homeostasis, interaction with platelets and fibronectin, increasing the fluid exudation and also growth factors.[7] Collagen can be found on the bone or skin of animals, such chicken shank.[8] The entire wound healing process is a complex series of events that begins at the moment of injury and can continue for months to years.[9]

Bone regeneration is a series of molecular and cellular process as well as tissue transformation. Bone defect healing process begins with inflammatory phase, which is vascular response to injury, requiring new vascularisation. Angiogenesis and osteogenesis are two closely correlated processes during bone regeneration vascularisation plays an important role in osteogenesis during the bone healing process.[10-11]

Vascular endothelial growth factor (VEGF) is an essential mediator during the process of angiogenesis that is an initial process of bone regeneration.

This research aims to determine the effects of chicken shank collagen scaffold on VEGF expression in bone regeneration. This research can be used as smart biomaterials that are able to act as the basis of modern therapies in dentistry.

Materials and methods

This research was a laboratory experimental research with post control group design. All animal care and experiments were performed under the institutional protocols approved by the Ethical Clearance Faculty of Dental Medicine Airlangga University (35/KKEPK.FKG/III/2015). Animals used were male rats (*Rattus norvegicus*) aged 3 months old and weighed 250 grams. Rats were allowed to acclimate for up to 7 days before inclusion in all experiments. Materials used in this research were chicken shank (SNI 7388.2009), a solution of acetic acid (CH_3COOH) and NaCl (Merck, Germany), VEGF polyclonal antibody (BIOSSUSA). Tools used in this research were a glass beaker, connicle tube, freezer (Royal Chest Freezer BD 195, China), magnetic stirrer (HANNA, USA), spatula glass, scales (Pioneer, USA), and freeze-dryer (Heto FD3, EN 87 164, Japan).

Fabrication of Chicken Shank Collagen Scaffold

Chicken shank was separated from the bone and cut into pieces, then blend. Chicken shank collagen was synthesized by adding trypsin powder and storage into incubator at a temperature of 37^0C for 24 hours. After that, chicken shank collagen was dissolved into a solution of acetic acid (CH_3COOH), and storage in 4^0C for 48 hours. Chicken shank collagen was blended with mixer until forming like fiber. It was sentrifuged with 9000 rpm for 10 minutes. Supernatant was separated by using micropipet and sentrifuged again with 9000 rpm for 10 minutes to obtained a pure supernatan. NaCl 5 wt% was added into supernatan until forming like a fiber. Fiber was dissolved with acetic acid 0,5 M and then added NaCl 5wt%. Fiber was dialyzed using cellophane membrane for 3 days, then was sentrifuged at 9000 rpm for 10 minutes.[3] Supernatant in the form of chicken shank collagen gel was inserted into the mold. The mold already containing chicken shank collagen 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.[12]

In vivo Transplantation

Research procedure was performed into several phases. Six rats were divided into two groups. Group 1 was the control group, while Group 2 was the treatment group.

The manufacture of bone defects was performed in both groups by drilling the rats' femoral sinistra 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, chicken shank collagen 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 2^{nd} week after the closure of the defect, those rats were anesthetized using 10% ether as asphyxiation, and then sacrificed. Those rats that had been sacrificed were then buried properly.[13]

Immunohistochemical Study

Tissue had been routinely formalin fixed (24–72 hours) and paraffin embedded. 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. The preparations were put in an incubator at a temperature of 37º C for 45 minutes. Next, the tissues were dripped with primary antibody Vascular Endothelial Growth Factor, and incubated at 4° C for one night. The tissues then were dripped with secondary antibody (biotinylation), and incubated at 37º 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.[14]

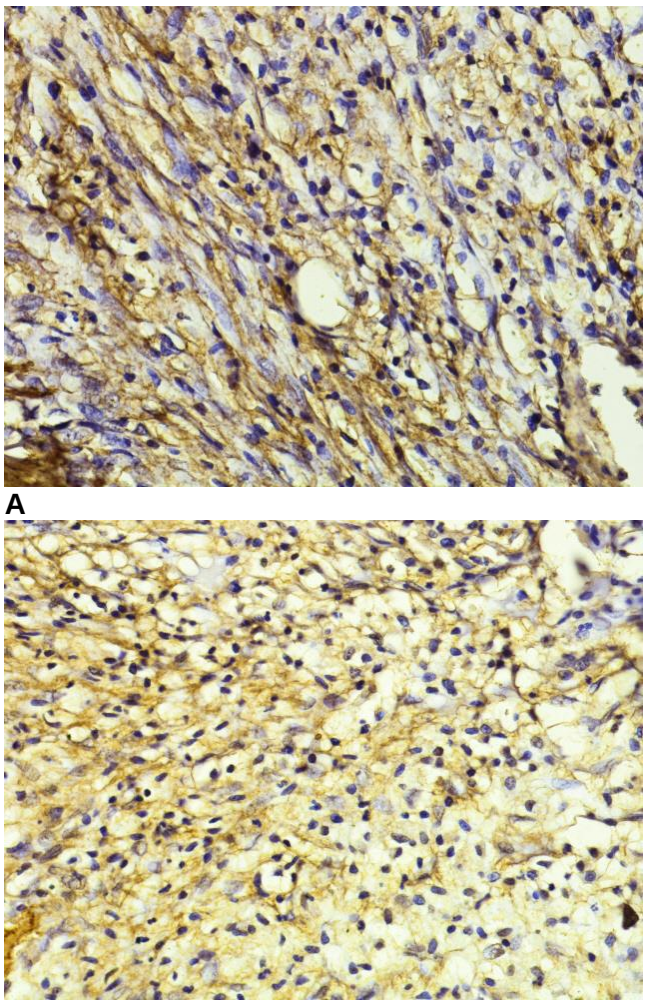
The expressions of VEGF were assessed semi-quantitatively using Remmele method. Remmele scale index (immuno reactive score/IRS) is the result of multiplying immuno-reactive cells percentage score to colour intensity score on the immuno-reactive cells. Data were obtained from the average value of the IRS in each sample observed in five different fields of view at 400x magnification. Those 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.

Statistical Analysis

Significant levels were determined by the Mann Whitney test. All statistical calculations were performed on the SPSS system for Windows (version 16.0, Statistical Package for Social Sciences (SPSS), Chicago, IL, USA). P-values of <0.05 were significantly considered.

Results

The VEGF distribution were show in figure 1. Based on the result of the statistical Mann Whitney test, p value obtained was more than 0,05 which means that there was a significant differences in VEGF expression between controp groups and treatment groups.



A

B

Figure 1. VEGF expression was distributed on control group (A) and the treatment group (B).

Discussion

This study presents a method of chicken shank collagen application in bone healing and regeneration for accelerating the proliferation of endothelial cells through the amount of VEGF expression.

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.[3]

Selection of biomaterials for scaffold design, thus, is essential to cell growth and proliferation in three-dimensional matrix. It is known that collagen, a natural biodegradable material, can support cellular ingrowth and matrix synthesis.[4]

In this research, showed that there was a significant difference in VEGF expressions between the treatment group and the control group on 2nd week during the process of bone regeneration. Chicken shank collagen could stimulate inflammatory cells and growth factors in the early phase of wound healing process. Chicken shank collagen contain RGD which binding integrin and its interaction with VEGF receptor. Integrin $\alpha_5\beta_1$ recognized by RGD binds to VEGF receptor.[15]

Bone regeneration can be affected by 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.[16] VEGF induces angiogenic process through endothelial cells. Bone-forming precursor cells migrate through the bloodstream to the callus that will differentiate into osteoblasts.[10] VEGF affects osteogenesis from day 14 to day 21 after the defect occurred.[17]

As natural biopolymers, collagen provide biologically specific signals for molecular interaction with the delivered cells and interact specifically with cells of the target tissue. Collagen contains basic residues, such as lysine and arginine, and specific cell adhesion sites such as argininеglycine-aspartate (RGD) groups. The RGD group actively induces cellular adhesion by binding to integrin receptors, and this interaction plays an important role in cell growth, and in the differentiation and overall regulation of cell functions.[6]

The specific cell binding amino acids of collagen for cell integrin receptors may be consumed. Collagen scaffolds showed a reduced mitogenic activity and structural integrity by degradation. The release of cytokine, VEGF, could have a positive effect on the early of bone healing. VEGF was sustainably released from the collagen scaffolds. After a burst release within few hours, VEGF release continued over as long as 28 days. Two weeks are generally proper to study the angiogenic effect of VEGF at a relatively early phase of tissue repair. This results may be achieved by increasing scaffold porosity, which may facilitate cell migration and blood vessel ingrowth into the scaffolds.[15]

Conclusions

Here we show that chicken shank collagen scaffold can increase VEGF expressions on the 2nd week during bone regeneration.

Declaration of Interest

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