

## Effect of rSLPI Amnion Membrane Application on Incision Wound of Rattus Norvegicus in Collagen and VEGF Expression

Elly Munadziroh<sup>1\*</sup>, R. Helal Soekartono<sup>1</sup>, Rossa Bella Vennowusky Rafli<sup>2</sup>,  
Anita Yuliaty<sup>1</sup>, Nadia Kartikasari<sup>3</sup>

1. Department of Dental Material, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
2. Undergraduate Student, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.
3. Departement of Prosthodontic, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

### Abstract

Amniotic membrane is one of the natural biomaterial that is widely used for wound healing which has Secretory Leukocyte Protease Inhibitor (SLPI) as an active component. One of rSLPI functions is suppressing several pro-inflammatory factors, such as serine protease. Suppression of serine proteinase in wound healing process may increase the amount of vascular endothelial growth factor (VEGF) and collagen.

The aim of this study is to evaluate the effect of rSLP amniotic membrane fluid application on incision wound of Rattus norvegicus in expression of VEGF and collagen.

The rats divided into 4 groups: control group and treated group with rSLPI (P1 0.03 cc, P2 0.045 cc, and P3 0.06 cc) (n=6). Incision was made on rat's cervical back. On the day 4, tissues around incision wound are collected for Masson's Trichrome (MT) and Immunohistochemistry (IHC) analysis. Expression of VEGF and collagen are calculated using microscope with 400x magnification.

Kruskal-Wallis test of VEGF showed significant value with  $p=0,006$  ( $p<0.05$ ). Mann-Whitney test of VEGF showed a significant difference between control and treatment group. Oneway ANOVA test result for collagen has  $p$  value 0.000 ( $p<0.05$ ). Honestly Significant Difference (HSD) test showed a significant differences between all group.

Application of rSLPI amniotic membran fluid in Rattus norvegicus incision wound may increase VEGF and collagen.

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### Introduction

The incision is one of basic procedure that used in oral treatment. Almost all of oral surgery procedure involves incision caused tissue damage. The body will recover through the wound healing process. Wound healing is a complex and dynamic process. Wound healing process is divided into three basic phases, hemostasis and inflammation, proliferation, maturation and remodeling<sup>1</sup>.

The proliferative phase may induce vascular network restoration and granulation tissue formation<sup>2</sup>. In this phase, vascular network restoration is important since nutrients and oxygen are needed during wound repair. Blood vessel formation (angiogenesis) is initiated by growth factors, vascular endothelial growth factor (VEGF)<sup>3</sup>. VEGF is a potent proangiogenic factor that has been shown to contribute 50% or more of the proangiogenic activity in wounds<sup>4</sup>. VEGF was increased within 24 hours until 7th day after the injury and then decreased significantly<sup>5</sup>. Proliferative phase starts from 4 - 20 days after the injury. In this phase, fibroblasts may form collagen and connective tissue. Collagen was detected on the day 3 after injury<sup>6,7,8</sup>.

Secretory Leukocyte Protease Inhibitor (SLPI) is member of acidic protein family. SLPI is an important key in epithelial tissue protection

#### \*Corresponding author:

Elly Munadziroh, DDS, M.Sc., Ph.D,  
Departement of Dental Material, Faculty of Dental Medicine,  
Universitas Airlangga, Jl. Mayjen Prof. Dr. Moestopo 47,  
Surabaya, Indonesia 60132  
E-mail: elly-m@fkg.unair.ac.id

from serine protease such as trypsin, leukocyte elastase, and cathepsin G<sup>2,9</sup>. SLPI has a function to accelerate wound healing<sup>2,10</sup>. Previous study showed application of SLPI in incision wound on 0.03 cc, 0.45 cc and 0.06 cc concentration may increase the number of macrophages and fibroblasts<sup>6</sup>. SLPI is finding in various body secretions such as seminal plasma, cervical mucus, breast milk, tears, saliva, and bronchial secretions<sup>2</sup>. Previous study showed amnion membrane had SLPI as active component<sup>10</sup>.

Physiologically, concentration of SLPI is very low, recombinant expression is required to produce sufficient amounts for detailed biochemical characterization and biomedical applications<sup>2</sup>. *Escherichia coli* has previously been utilized as a host for heterologous production of recombinant SLPI (rSLPI)<sup>2,10</sup>. The aim of this study is to analyze the effect of rSLP amniotic membrane fluid application on incision wound of *Rattus norvegicus* in expression of VEGF and collagen on the day 4.

## Materials and methods

### Experimental Design

This study was an experimental laboratory using post test only control group design. This study was conducted in experimental animals using *Rattus norvegicus* as sample. This research had been approved with ethical clearance from Committee of Ethical Clearance of Health Research, Faculty of Dental Medicine, Universitas Airlangga, number 270/KKEPK.FKG/IX/2015. Criteria for samples are male *Rattus norvegicus* 2-3 months with 250-300 gram weight. rSLPI obtained from Proteomic Laboratory from Institute of Tropical Disease, Universitas Airlangga.

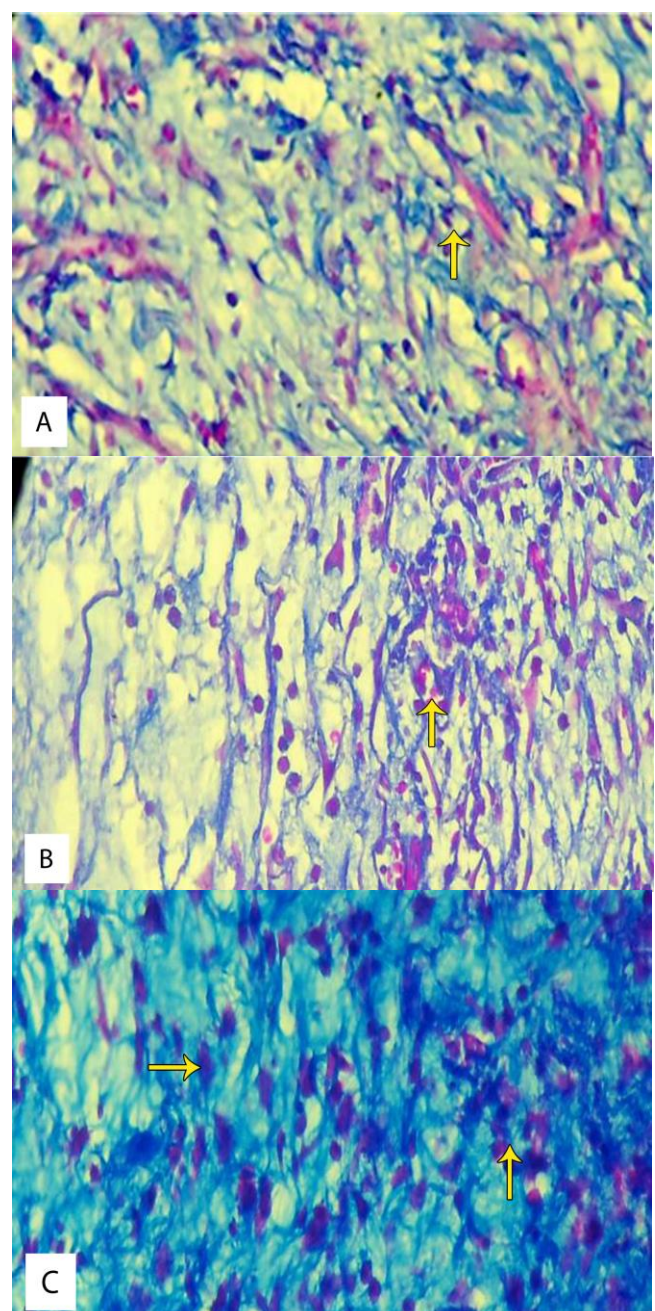
### Animal Treatment

Before the experiment begins, the rat was given adaptation in a cage (60 cm x 65 cm x 80 cm) for a week. The rats divided into 4 groups: control group and treated group with SLPI (P1 0.03 cc, P2 0,045 cc, and P3 0.06 cc). Each group consists of 6 rats. Before incision wound made, rat's hair has been removed. Incision was made on rat's cervical back with length 1 cm and deep 1 cm under the effect of anaesthesia intramuscular of ketamine (Kepro, Daventer, Holland) with dose 3-5 mg/kg BB. Histopatologic analysis (HPA) is conducted using immunohistochemistry (IHC) staining for VEGF

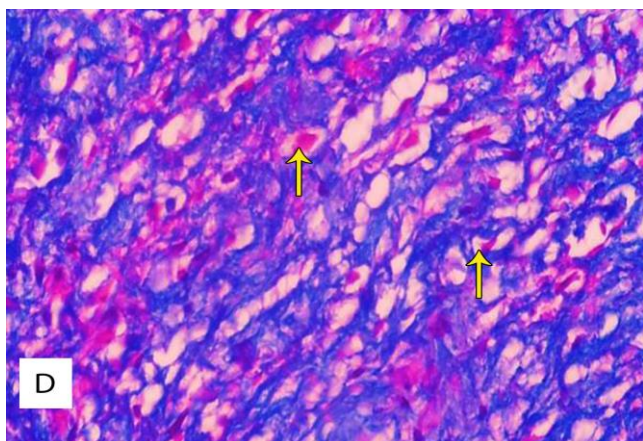
(Bioss, Massachusetts, USA) and Masson's Trichrome (MT) staining for collagen. HPA preparation was observed with 400x magnification to measure the expression of VEGF and collagen. The obtained data was analyzed by Kruskal-Wallis test for VEGF and Oneway ANOVA for collagen.

## Results

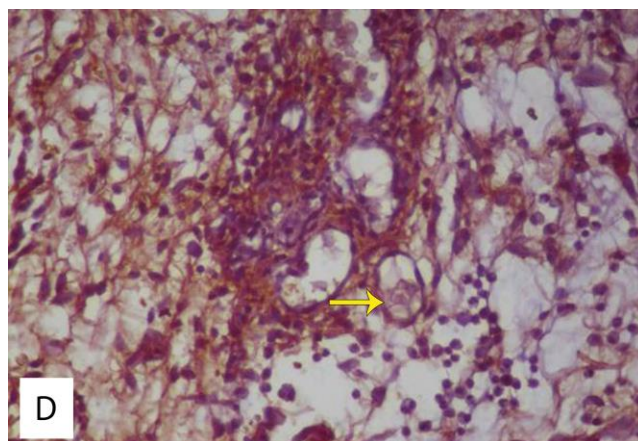
Expression of VEGF could be seen in Figure 1 while collagen expression could be seen in Figure 2.



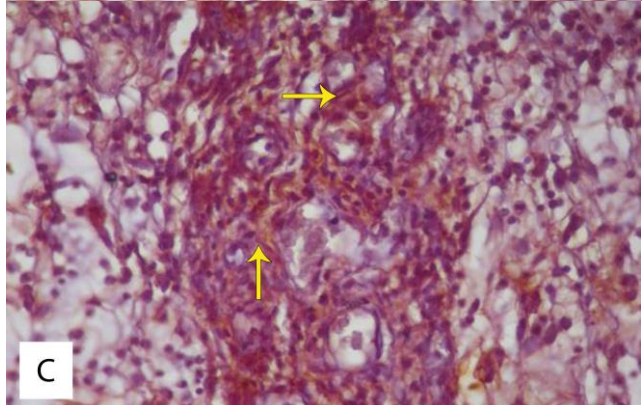
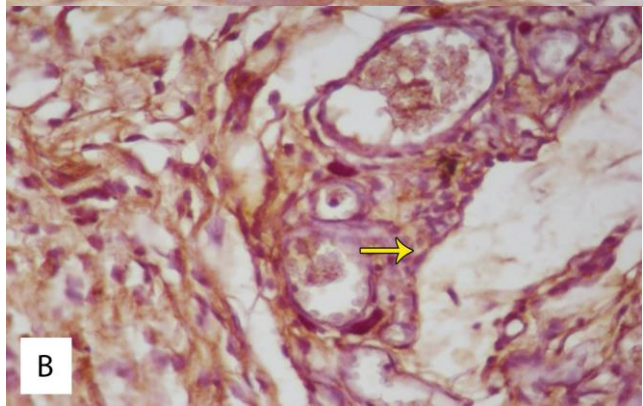
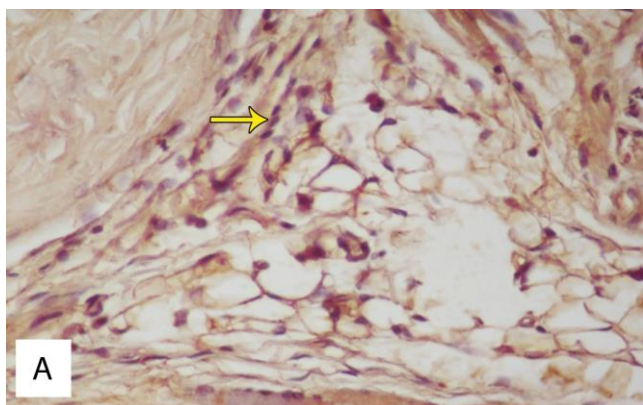




**Figure 1.** VEGF expression (yellow arrow) in light microscope 400x magnification in control (a), P1 (b), P2 (c), and P3 (d) group.



**Figure 2.** Collagen expression (yellow arrow) in light microscope 400x magnification in control (a), P1 (b), P2 (c), and P3 (d) group.



The average and standard deviations (SD) between the amount of VEGF and collagen from control group and the treatment group in wounded can be seen in the Table 1.

Group	n	VEGF		Collagen	
		Mean	SD	Mean	SD
Control	6	11,83	1,169	0,83	0,408
P1	6	13,67	1,966	0,00	0,000
P2	6	20,17	5,742	2,17	0,408
P3	6	21,67	3,933	3,00	0,632

**Table 1.** Mean and standard deviation of VEGF dan collagen.

Data of VEGF had normal distribution and heterogen. Due to this result, Kruskal-Walli's test should be conducted. The result of Kruskal-Walli's test showed that p value is 0.006 with significance level  $\alpha = 5\%$ . Differences between treatment groups was analyzed by Mann-Whitney shows in Table 2.

Group	Contro			
	I	P1	P2	P3
Control	-	0,059	0,015(*)	0,006(*)
P1	-	-	0,076	0,036(*)
P2	-	-	-	0,422
P3	-	-	-	-

(\*) = Significant level ( $p < 0,05$ )

**Table 2.** Results of Mann-Whitney Test of VEGF.

Oneway ANOVA test was performed for the data of collagen. The results showed p value

is 0.000 ( $p < 0.05$ ). Differences between groups of collagen was analyzed using Tukey HSD (Honestly Significant Difference). The result of HSD test can be seen in Table 3.

Group	Control	P1	P2	P3
Control	0,015(*)	0,015(*)	0,00(*)	0,00(*)
P1	-	-	0,00(*)	0,00(*)
P2	-	-	-	0,015(*)
P3	-	-	-	-

(\*) = Significant level ( $p < 0,05$ )

**Table 3.** Tukey HSD Test of Collagen.

### Discussion

SLPI is a multifunctional protein in the host defense response. SLPI is an importance factor which regulates the innate and adaptive immunity. SLPI may protect our body through several mechanisms (i) as an antimicrobial agent, (ii) as a controller of inflammatory mediators and protects the host from excessive tissue damage by proteolytic enzymes released during inflammation, (iii) as a suppressor of inflammatory responses, (iv) regulates the production and pro-immunogenic function of neutrophil extracellular traps, and (v) it fosters repair and is a component of the molecular machinery that controls cell growth, differentiation and apoptosis. The main function result of SLPI actions in wound healing process is to counteract excessive inflammatory responses and to initiate healing processes<sup>9,10</sup>.

Wound healing process is a complex process which divided into several phases (inflammation, proliferation, and remodeling)<sup>11,12</sup>. SLPI may also play an important role in all phases of wound healing process. Previous study showed that SLPI-null mice have prolonged plasma clotting time<sup>13</sup>. Another research also shown that cutaneous wound without exogenous application of SLPI may delay in skin wound healing<sup>12,13</sup>.

The result of this study showed that VEGF expression is increased with the increasing concentration of rSLPI. VEGF is produced by macrophages and plays an important role for angiogenesis<sup>10</sup>. This might be happened due to application of rSLPI might decrease pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ), and tumor necrosis factor

beta (TNF- $\beta$ ). Previous research showed that the absence of SLPI may increase elastase activity, excessive and prolonged inflammation, enhanced activity (but not expression) of TNF- $\beta$ , and elevated expression of TNF- $\alpha$ <sup>12,13,14</sup>.

In this study, collagen levels increased along with increasing concentration of rSLPI. Increased collagen expression might happen due to SLPI being able to inhibit elastase. Elastase may decrease variety of proteins such as collagen and fibronectin. In addition, elastase also decreases Growth Factor (GF) and cytokine. Fibroblasts migrate to the injured area then increase of collagen amount. Fibroblasts produce extracellular matrix and replaced by collagen. Collagen will form a new network and wound healing process start<sup>10,14,15</sup>.

SLPI stimulate the formation of Hepatocyte Growth Factor (HGF). HGF is a cytokine that produced by mesenchymal cells. These cells play an important role for the regulation of mitogenesis, motogenesis and morphogenesis. In addition, SLPI also affect fibroblast ability to contract collagen gels. Contraction of collagen gel was the result of fibroblast to organize and solidify the collagen fibers<sup>10,13,16</sup>.

### Conclusions

Application of rSLPI amniotic membrane fluid in *Rattus norvegicus* incision wound may increase VEGF and collagen in wound healing process.

### Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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