
ORIGINAL ARTICLE**Role of Lactoferrin Fibroblast Growth Factor 2 and Vascular Endothelial Growth Factor in Gingival Wounds***Istiaty^{1*}, Intan Nirwana², Indra Surjono¹, Meircurius Dwi Condro Surboyo³**¹Department of Oral Pathology and Maxillofacial, ²Department of Dental Material, ³Department of Oral Medicine, Faculty of Dental Medicine. Universitas Airlangga, Surabaya, Indonesia***Abstract:**

Background: Lactoferrin possesses the ability to promote migration and proliferation of fibroblasts which represents one potential reason for the use of lactoferrin to accelerate the healing process in gingival wounds. *Aim and Objectives:* To analyze the role of lactoferrin in Fibroblast Growth Factor 2 (FGF-2) and Vascular Endothelial Growth Factor (VEGF) expression in the healing process of gingival wounds. *Material and Methods:* Twenty eight male Wistar rats were divided into four treatment groups. All rats were incised using a full thickness method on the gingival anterior region of the mandible to which a single application of lactoferrin concentration 10 µg/ml, 20 µg/ml, 40 µg/ml and phosphate buffer saline (control) was made. On the third day post-application, the rats were sacrificed to enable removal of the gingival tissue on the mandible. The role of lactoferrin was evaluated based on FGF-2 and VEGF expression identified through immunohistochemical staining. *Results:* In general, the group administered with lactoferrin showed higher FGF-2 and VEGF expression compared to that of the control group (p=0.000). The group treated with 40 µg/ml lactoferrin concentration showed a higher FGF-2 and VEGF expression than the groups treated with 10 µg/ml and 20 µg/ml (p=0.000) lactoferrin concentration. *Conclusion:* Lactoferrin concentration of 40 µg/ml can accelerate the healing process of gingival wounds by increasing FGF-2 and VEGF expression.

Keywords: Lactoferrin, Fibroblast Growth Factor 2, Vascular Endothelial Growth Factor, Gingival Wound

Introduction:

Lactoferrin, an iron-binding glycoprotein member of the transferrin family, is synthesized by epithelial cells and secreted into body fluids such as colostrum, saliva, tears and mucin. The widespread distribution of lactoferrin in the digestive, respiratory and reproductive systems shows that it plays an important role in the nonspecific host defenses of mammals [1].

Lactoferrin constitutes the main component of polymorphonuclear neutrophil granules and is released into plasma during infection or inflammation. Lactoferrin induces Th1 (T-helper1) to produce cytokines and increases the phagocytic activity of macrophages and natural killer cells, while also promoting both the maturation of dendritic cells and hydroxyl radical production by neutrophils. This property is important to the inflammatory phase in the healing process of epithelial wounds [1].

Lactoferrin plays a role in the wound healing process based on its ability to promote the migration and proliferation of fibroblasts. Lactoferrin has a synergistic effect on Fibroblast Growth Factor-2 [2]. Vascular Endothelial Growth Factor and FGF-2 are growth factors active in the angiogenesis process during wound healing. Human apo-lactoferrin and bovine lactoferrin have contrasting effects on this process. Human apo-lactoferrin promotes angiogenesis by

stimulating VEGF-A [3], thereby increasing the proliferation and migration of VEGF from Human Umbilical Vein Endothelial Cells (HUVECs) by intensifying Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) expression [4]. In addition, a study conducted by Zhang *et al.* (2014) showed that the administering of lactoferrin concentrations of 50-100 µg/ml can increase human tenocyte cell survival in serum-free culture medium and promote the proliferation and synthesis of human tenocyte in 1% serum bovine fetal culture medium [5].

Periodontal surgery procedures such as gingivectomies, operculectomies and curettage produce a disintegration of epithelial tissue, referred to as a wound. To promote the wound healing process and avoid infection resulting in disrupted healing in gingival and periodontal tissue, periodontal dressing is applied after surgery. Periodontal dressing has anti-bacterial, anti-coagulant, growth factor-stimulating and analgesic properties [6]. At present, there are no periodontal dressings that contain lactoferrin. Therefore, based on the potential role of lactoferrin in the inflammatory process and its ability to stimulate growth and cell proliferation factors, it is necessary to confirm the function of lactoferrin in the gingival wound healing process by analyzing the expression of VEGF and FGF-2.

Material and Methods:

The research project received ethical clearance number: 198/HRECC.FODM/VII/2018 from the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga.

Animals

The research project consisted of three month-old male Wistar rats (*Rattus norvegicus*) weighing

235-252 g. Twenty-eight rats were placed in individual cages at room temperature and acclimated for seven days before being treated. During acclimation, they were provided with food and drink in accordance with the standard rules of animal testing.

Gingival wounds

Gingival wounds were produced according to the method used by Caceres *et al.* (2014). They were made by means of a full thickness incision measuring 3 mm × 1.5 mm in the anterior region of the mandibular gingiva (the gingiva between the first incisive dextra and sinistra from the gingiva margin to the mucogingival junction) using a stainless steel, crescent-shaped, surgical blade (No. 15, OneMed) with the support of periodontal probes (BPCP2, Osung MND, Korea) [7]. During wound production, the rats were anesthetized with a ketamine/xylazine cocktail.

Application of lactoferrin to the gingival wound

The lactoferrin used in this study was bovine lactoferrin (Lactoferrin from bovine milk, L9507, Sigma Aldrich) diluted with phosphate buffer saline (P3813, Sigma Aldrich) to produce concentrations of 10 µg/ml, 20 µg/ml and 40 µg/ml. The application of lactoferrin to the gingival wound in the anterior region of the mandible involved the intraoral dripping method as described by Hitomi *et al.* (2015) and was conducted immediately after creation of the wound [8-9]. The experimental subjects were divided into four groups, namely:

Group A (control group): Phosphate buffer saline applied

Group B: Lactoferrin concentration 10 µg/ml applied

Group C: Lactoferrin concentration 20 $\mu\text{g/ml}$ applied

Group D: Lactoferrin concentration 40 $\mu\text{g/ml}$ applied

FGF-2 and VEGF expression in gingival wounds

Three days after treatment, each rat having been terminated, their anterior gingival mandible was subjected to biopsy before immunohistochemistry staining to determine the FGF-2 expression (FGF anti-antibody monoclonal, ab9588, Abcam) and VEGF (VEGF anti-antibody monoclonal, ab1316, Abcam). All measurements were taken using a light microscope (Nikon H600L microscope; Nikon, Japan) at a magnification of 100 \times and 400 \times (DS Fi2 300MP digital camera; Nikon, Japan, software digital imaging by Nikon Image System, Nikon, Japan).

Statistical Analysis:

Data were expressed as the mean values \pm Standard Deviation (SD) for each measurement and analysed by One Way ANOVA and post hoc test LSD with a significant number $p < 0.05$ using SPSS 24.0 for Windows.

Results:

Microscopic analysis of gingival wounds in the anterior regional gingiva of the mandible revealed disintegration of the gingival tissue, although the disintegration only occurred in the lamina propria and the rete pegs appeared to be have remained intact (Fig.1).

VEGF expression was observed in the capillaries which had formed in the wound area (Fig. 2). In general, the group to which lactoferrin had been applied showed higher VEGF expression compared to that of the control group. The group treated with 40 $\mu\text{g/ml}$ lactoferrin concentration showed higher VEGF expression compared to the group to which 20 $\mu\text{g/ml}$ and 10 $\mu\text{g/ml}$ ($p = 0.00$) lactoferrin concentration had been applied. The group treated with 10 $\mu\text{g/ml}$ lactoferrin concentration expressed the same level of VEGF in comparison with the group to which 20 $\mu\text{g/ml}$ lactoferrin concentration ($p = 0.78$) had been applied (Fig. 3).

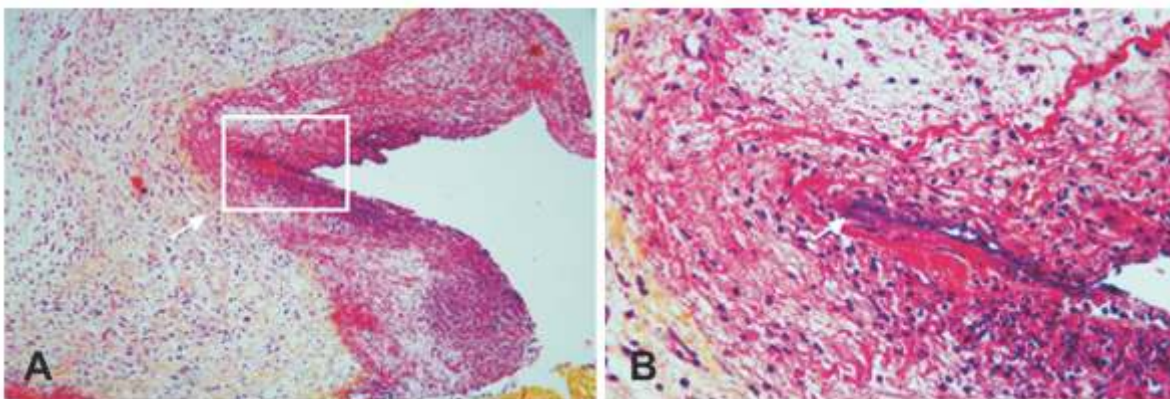


Fig.1: Microscopic Picture of Gingival Wounds: (A) Disintegration of Gingival Tissue and Complete Retepeg (100 \times Magnification), (B) Epithelium Presents No Disintegration up to the Lamina Propria (400 \times Magnification)

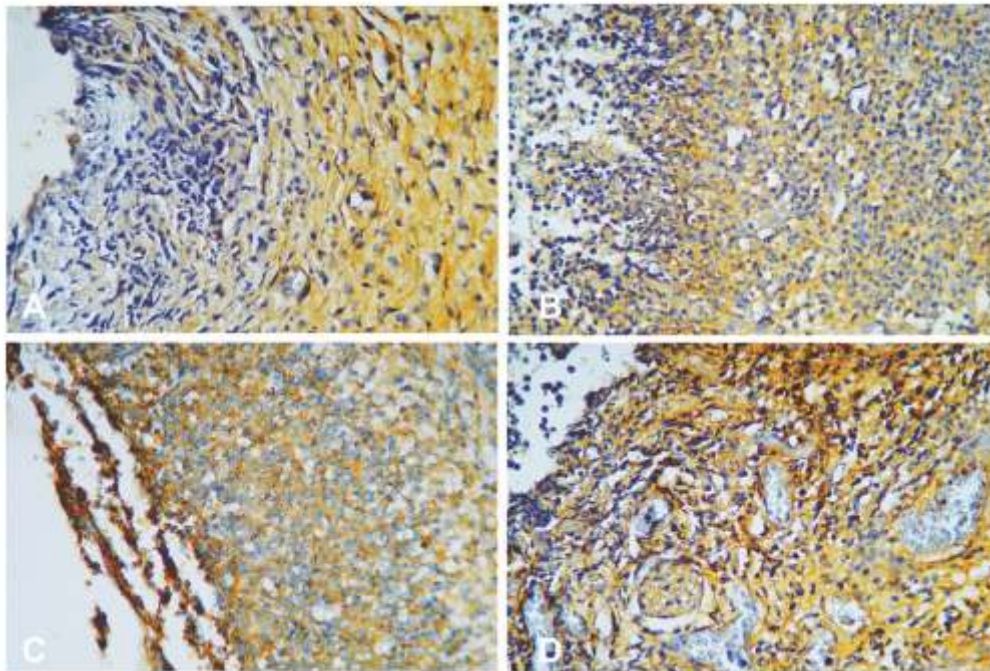


Fig. 2: VEGF Expression in Gingival Wounds (400x magnification). Control Group (A); Group Treated with 10 µg/ml of Lactoferrin (B); Group Treated with 20 µg/ml of Lactoferrin (C); Group Treated with 40 µg/ml of Lactoferrin (D).

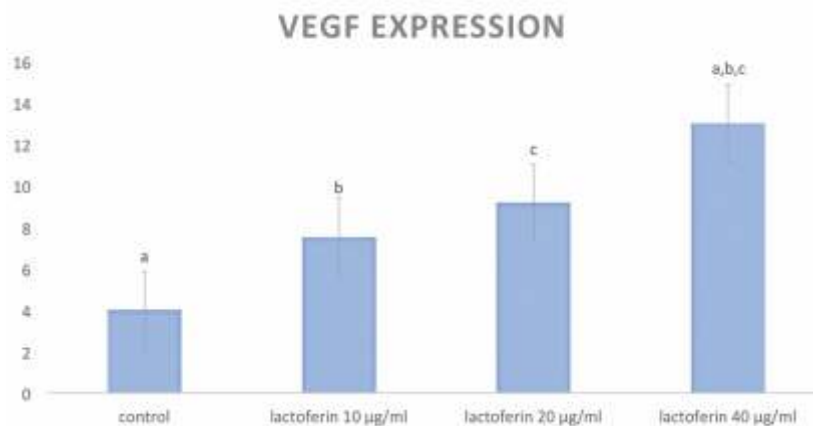


Fig. 3: VEGF Expression. Same Letters Show Significant Differences between Groups (p <0.05).

FGF-2 expression was observed in the wound area, the lowest level being that of the control group (Fig. 4). Overall, the group applied with lactoferrin showed higher FGF-2 expression compared to the control group. The group treated with 40 µg/ml lactoferrin concentration showed higher FGF-2 expression compared to the group

to which 20 µg/ml and 10 µg/ml (p=0.00) lactoferrin concentration had been applied. The group treated with 10 µg/ml lactoferrin concentration expressed the same level of FGF-2 as the group applied with 20 µg/ml lactoferrin concentration (p=0.417) (Fig. 5).

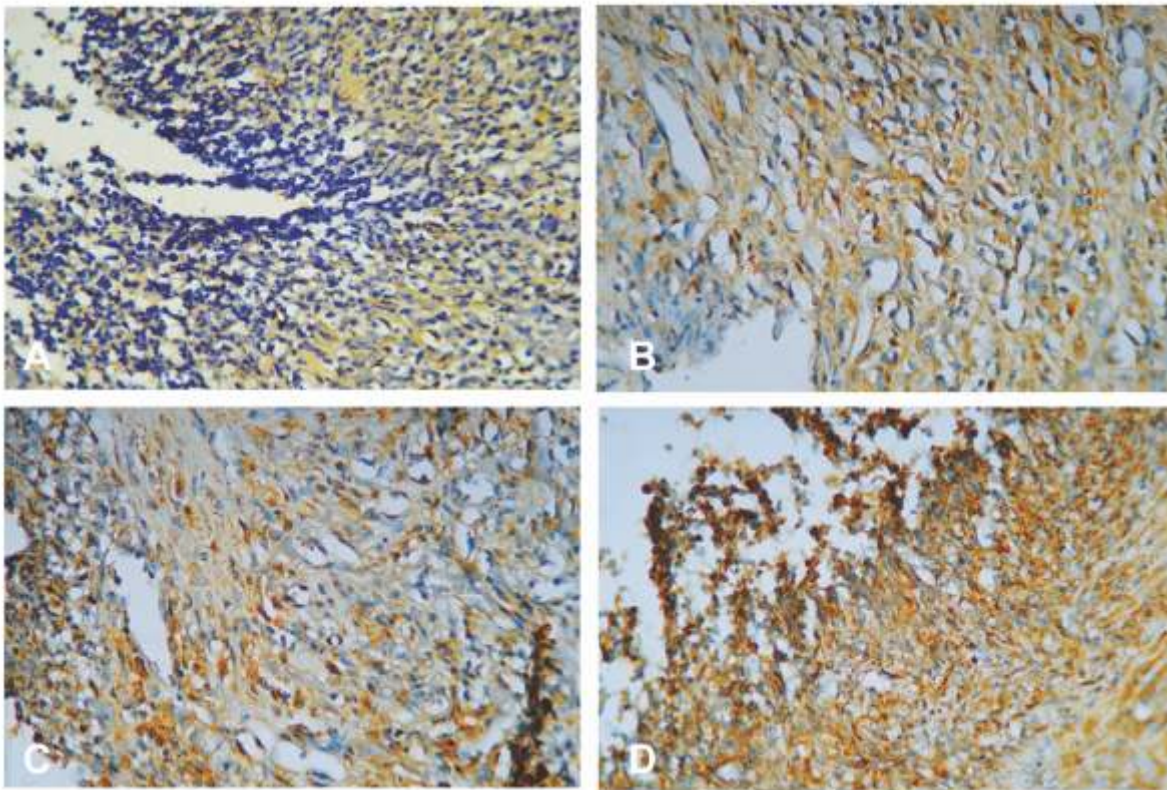


Fig. 4: FGF-2 Expression in Gingival Wounds (400 × Magnification). Control Group (A); Group Treated with 10µg/ml of Lactoferrin (B); Group Treated with 20 µg/ml of Lactoferrin (C); Group Treated with 40 µg/ml of Lactoferrin (D).

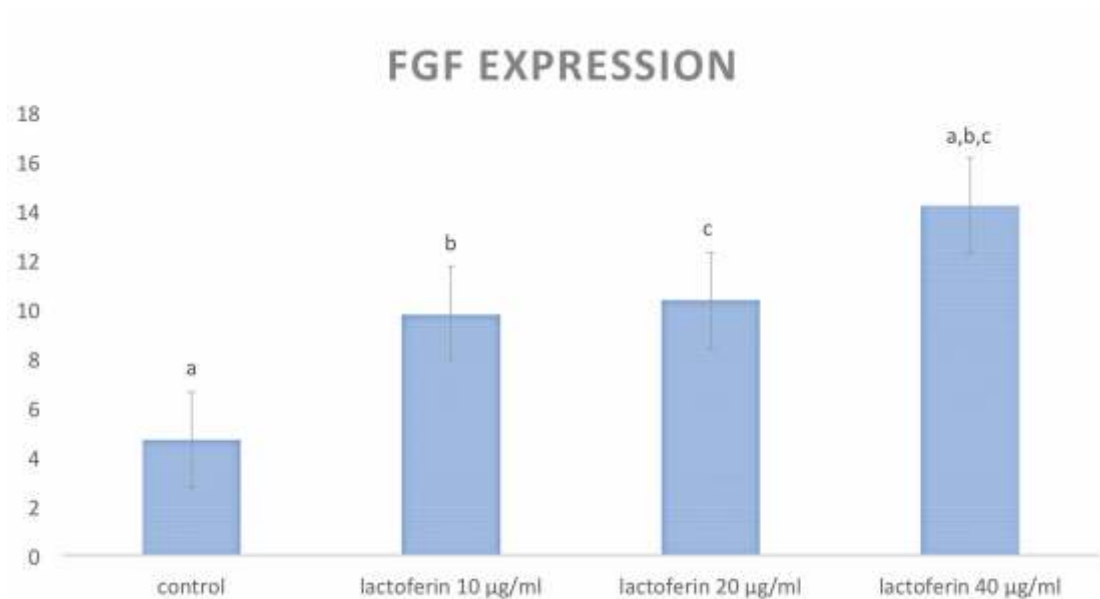


Fig. 5: FGF-2 Expression. Same Letters show Significant Differences between Groups (p <0.05).

Discussion:

The application of lactoferrin at a concentration of 40 µg/ml to gingival wounds showed the highest expression of VEGF and FGF-2 when compared with the group treated with lactoferrin concentrations of 20 µg/ml and 10 µg/ml. These results are in accordance with those produced by the study conducted by Anand *et al* (2015) which reported that administration of lactoferrin concentrations of 10-40 µg/ml can increase the production of Reactive Oxygen Species (ROS) associated with increased phagocytic activity in macrophages [9]. This increase in phagocytic activity plays an important role in wound healing. Macrophages are formed from monocytes stimulated by extracellular matrix protein fragments, Transforming Growth Factor β (TFG-β) and Monocyte Chemoattractant Protein 1 (MCP-1). Macrophages also secrete pro-inflammatory enzymes and cytokines such as collagenase, which stimulates the proliferation of fibroblasts and promote angiogenesis, and transforming growth factor [10].

There are two mechanisms that possibly explain how lactoferrin increases the expression of VEGF and FGF-2 in the gingival wound healing process in this study. Firstly, lactoferrin plays a role in the epithelial mucosa and inflammatory cells because it can also bind to the Scavenger Receptor C-Type Lectin (SRCL) extensively expressed by endothelial cells [11]. SRCL is the main receptor for lactoferrin both locally in the site of inflammation and systemically. The binding of lactoferrin to these receptors activates innate immune cells through the mechanism of motor motility, superoxide production and the release of molecules and proinflammatory cytokines such as nitrous oxide, TNF-α and IL-8. Secondly,

lactoferrin is known to be a chemoattractant for PMN, monocytes, murine myeloid cells and dendrite cells [12].

Lactoferrin demonstrates the ability to induce production of TNF-α, IL-8 and NO by macrophages. TNF-α is the first cytokine produced when administration of lactoferrin is followed by secretion of IL-8. This condition can be explained by the fact that TNF-α is the first cytokine produced in the cytokine cascade which causes the production of other cytokines [13]. Production of TNF-α by macrophages, which then induces IL-8 production by keratinocytes, and superoxide activation by endothelial cells causes increased production of VEGF by endothelium and production of FGF-2 by keratinocytes. Both of these processes also result in an increase in the proliferation stage of the gingival wound healing process.

Lactoferrin enhances the ability of macrophages and dendrite cells to stimulate T cells and modulate inflammatory responses. Research conducted by Hwang (2016) showed that administration of lactoferrin on CD14⁺ can increase the production of IL-10, IL-6 and MCP-1. In contrast, treated CD16⁺ showed an increase in IL-12p40, IL-10 production and reduced TNF-α production. Lactoferrin can also influence antigen-presenting expression and costimulatory molecules [14]. Macrophage cell surfaces express presenting antigens and costimulatory molecules responsible for antigen presentation and activation of receptive T cells, initiation and expansion of memory. Human Leukocyte Antigens (HLA-A, HLA-B, HLA-C) present antigens to CD8⁺ cells, while HLA-DR presents antigens to CD4⁺ cells. Molecules of CD80 and CD86 co-stimulators are

secondary signals needed for Tcell activation during antigen presentation. The administering of lactoferrin can increase the expression of CD86 and CD1c on CD14 +, whereas on CD16 + it can increase the expression of CD86 and HLA-A, HLA-B, HLA-C [14].

The administering of lactoferrin to gingival wounds induces a decrease in the production of proinflammatory cytokines by macrophages and increased expression of VEGF and FGF-2. Lactoferrin does not have special receptors on macrophage cells. Lactoferrin can bind to Receptors for Advanced Glycation End Products (RAGE) on the surface of macrophage cells. The bond between lactoferrin and RAGE results in a decrease in the formation of ROS which is a compound playing an important role in NFκB signalling. This decrease in the formation of ROS results in a decrease in phosphorylation and activation of NFκB. Decreased proinflammatory cytokine production, increased VEGF expression and greater expression of FGF-2 can accelerate the gingival wound healing process [15].

Periodontal surgery such as gingivectomy, operculectomy and curettage involve the use of periodontal dressings which contain anti-bacterial, anti-coagulant, stimulating growth factors and analgesics. Periodontal dressing

content is divided into three types, namely; that containing zinc oxide and eugenol, that containing zinc oxide without eugenol or that containing neither zinc oxide nor eugenol. The periodontal dressing function minimizes the risk of postoperative complications such as infection and bleeding, while also improving tissue healing by preventing physical trauma during mastication [6]. Periodontal dressing serves only to reduce pain and discomfort after periodontal surgery, having no therapeutic effect as mentioned above. This is supported by research conducted by Soheilifar et al. (2015) which demonstrated that post-surgical periodontal dressing merely reduces the pain experienced by the patient. As far as bleeding and swelling of the gingiva are concerned, post-surgical periodontal dressing is no greater benefit to the patient than not applying such dressing [16].

Conclusion

The results of this study confirm that a lactoferrin concentration of 40 µg/ml can accelerate the healing process of gingival wounds by increasing FGF-2 and VEGF expression. This result can also underpin the development of periodontal dressings containing lactoferrin.

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