

The increase of VEGF expressions and new blood vessels formation in Wistar rats induced with post-tooth extraction sponge amnion

Moh. Basroni Rizal,¹ Elly Munadzihroh,² and Indah Listiana Kriswandini³

¹ Department of Dental Material, Faculty of Dentistry, Universitas Hang Tuah

² Department of Dental Material, Faculty of Dental Medicine, Universitas Airlangga

³ Department of Oral Biology, Faculty of Dental Medicine, Universitas Airlangga
Surabaya - Indonesia

ABSTRACT

Background: Tooth extraction is the process of removing a tooth from the oral cavity potentially triggering a wound healing response in the body. As a result, many methods have been applied to improve the wound healing process, especially in wounds resulting in complications. One such method involves the application of amniotic membrane which has anti-inflammatory, anti-bacterial, anti-fibrosis, anti-scarring properties with low immunogenicity, epithelialization effects, and secretory leukocyte protease inhibitor (SLPI). It also contains collagen, various growth factors, transferrin, fibronectin, nidogen, proteoglycans, hyaluronan and laminin. **Purpose:** This study aimed to determine the effects of sponge amnion on the number of VEGF expressions and new blood vessels in post-tooth extraction wounds of Wistar rats. **Methods:** Sponge amnion was produced by mixing freeze-dried amnion membrane from the Tissue Bank at RSUD Dr. Soetomo with 1% gelatin before freeze drying the mixture. Wistar rats were then divided into two groups. In Group 1, referred to as the control group, the post-extraction wounds of the rats received no treatment. Meanwhile, in Group 2, the treatment group, the subjects' post-extraction wounds were treated with sponge amnion. The rats of both groups were sacrificed on day 3 to allow observation of the number of VEGF expressions and new blood vessels. A statistical analysis test, a t-test, was subsequently conducted. **Results:** There was a significant difference in the number of new blood vessels in the control group and that of the treatment group with a p value of 0.018 ($p < 0.05$). There was also a significant difference in VEGF expression between the two groups with a p value of 0.000 ($p < 0.05$). **Conclusion:** Sponge amnion can generate a number of VEGF expressions and new blood vessels in the post-extraction wounds of Wistar rats.

Keywords: sponge amnion; angiogenesis; socket healing; VEGF expressions

Correspondence: Elly Munadzihroh, Department of Dental Material, Faculty of Dental Medicine, Universitas Airlangga. Jl. Mayjend. Prof. Dr. Moestopo no. 47 Surabaya 60132, Indonesia. E-mail: emunadzihroh@yahoo.com; m.basroni.rizal@gmail.com;

INTRODUCTION

Tooth extraction is a common procedure performed by dentists with a prevalence rate in Indonesia of 38.5%.¹ Nevertheless, the process can lead to complications in 37.6% of cases. Fractures are the most common complication at 30.4% prevalence. The tooth extraction socket may be considered as a form of bone fracture which can cause disruption to the wound healing process, possibly triggering a wound healing response from the body.²

In general, the wound healing process can be divided into three phases, namely; inflammatory, proliferative and remodeling.³ The inflammatory phase initiates a condition

in which macrophages secrete cytokines and regulatory factors. These consist of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and transforming growth factor-beta1 (TGF- β 1), all of which play an important role in the wound healing process.⁴ New blood vessels formed through a process of angiogenesis play a role in maintaining the continuous function of various tissues and organs affected by the wound⁵ by subsequently supplying oxygen and nutrients useful for the formation of new tissues.⁶

Currently, various methods of improving wound healing have been implemented, one of which involves placing an amniotic membrane on the wound. Amniotic membrane

is known to contain collagen types I, III, IV, V and VI, fibronectin, nidogen, proteoglycans, hyaluronan, laminin and secretory leukocyte protease inhibitor (SLPI).^{7,8} Moreover, it also contains various growth factors, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF) and transforming growth factor (TGF- α).⁹ In fact, amniotic membranes have widely been employed as biomaterials within various clinical applications due to the fact that it is flexible and semi-transparent, protects the wound, reduces pain and has a re-epithelization effect.^{7,10,11} Therefore, this research aimed to assess the application of amnion sponge on post-extraction wounds by observing the number of VEGF expressions and new blood vessels formation as indicators of the angiogenesis process.

MATERIALS AND METHODS

This research constituted a laboratory-based experimental investigation using ten male Wistar rats weighing 200–250 grams. The subjects were obtained from the Biochemistry Laboratory of the Faculty of Medicine, Universitas Airlangga. They were then divided into two groups of five. In group 1, referred to as the control group, the Wistar rats' post-tooth extraction wounds remained untreated. Meanwhile, in group 2, known as the treatment group, such wounds were given sponge amnion.

The Wistar rats were anesthetized intramuscularly, their mandibular incisors subsequently being extracted using lower anterior forcep. In the control group, suturing was conducted post-tooth extraction. Meanwhile, in the treatment group, after extraction, amnion sponge was applied to the extraction sockets, before suturing was performed.

Sponge amnion was produced by smoothing freeze-dried amnion membranes (<24 hours) from Biomaterials Center / Tissue Bank / Dr. Soetomo Hospital with a sterile aquadest at a ratio of 1: 1. After becoming amniotic porridge, it was added to 1% gelatin at a ratio of 1:1, and

then freeze dried (Lyophilizer) for 2 x 24 hours to induce the formation of sponge. On the third day, both groups of Wistar rats were sacrificed. Their mandibular tissue, together with the extraction socket, was removed and soaked in fixation solution for 48 hours. The two were then decalcified until the bone became sufficiently tender to be cut.

Thereafter, paraffin blocks were prepared and cut to a thickness of 4–5 μ . Histologic preparations were made incorporating the use of both immunohistochemical imaging (IHC) to enable observation of VEGF expression and hematoxylin-eosin (HE) staining to highlight the number of new blood vessels. Data calculations were then performed under a light microscope at 400x magnification on the 1/3 apical socket area of the tooth extraction. The VEGF expressions measured were the number of endothelial cells emitted a brownish color on immunohistochemical staining with anti-VEGF polyclonal antibodies (abcam product, ab46154). The number of blood vessels, on the other hand, was determined by the prevalence of luminal formations surrounded by a layer of endothelial cells. The data obtained was then analyzed by means of a parametric test using an independent t-test with a significance level of 95% (0.05).

RESULTS

The results of this research confirmed that VEGF expressions in one third of the tooth extraction apical sockets of the treatment group were higher than those in the control group. Figure 1 illustrates VEGF expressions after IHC staining on one third of the tooth extraction apical sockets at a magnification of 400x.

Moreover, the results indicated that the number of new blood vessels in one-third of the tooth extraction apical sockets of the treatment group were higher than those of the control group. Figure 2 demonstrates that the appearance of a lumen-formed image surrounded by a layer of endothelial cells in one-third of the tooth extraction apical socket preparations after HE staining at a magnification of 400x.

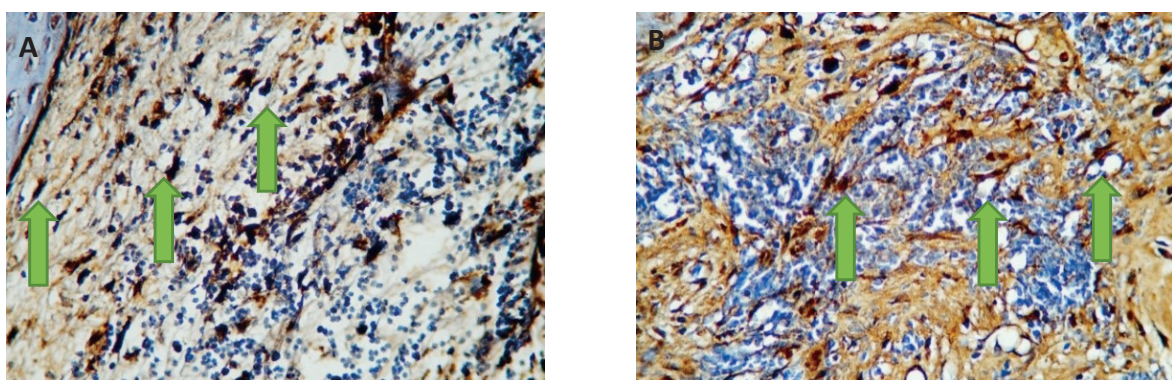


Figure 1. VEGF Expressions. (a) VEGF expressions in the control group on day 3. (b) VEGF expressions in the treatment group on day 3. Arrows show the endothelial cells expressing VEGF (brown color) after the IHC staining at a magnification of 400x.

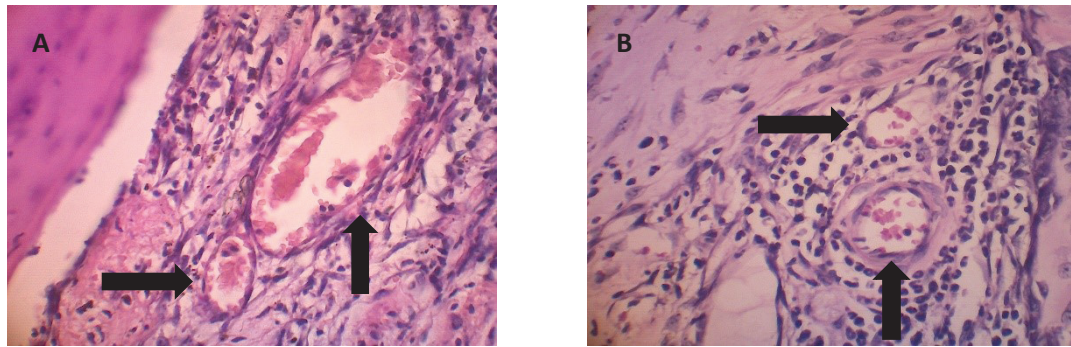


Figure 2. The number of new blood vessels. (a) The number of new blood vessels in the control group on day 3. (b) The number of new blood vessels in the treatment group on day 3. The arrows show the lumen of the new blood vessels after the HE staining at a magnification of 400x.

Table 1. Distribution of normality

	VEGF expressions	Number of new blood vessels
Number of samples	10	10
Kolmogorov-Smirnov Z	0.634	0.704

Table 2. The mean, standard deviation, and p value of VEGF expressions and new blood vessels in the control and treatment groups

Groups	VEGF expressions	Number of new blood vessels
Control	3.60 ± 0.89	7.60 ± 2.07
Treatment	11.80 ± 2.05	12.60 ± 3.13
P Value	0.000	0.018

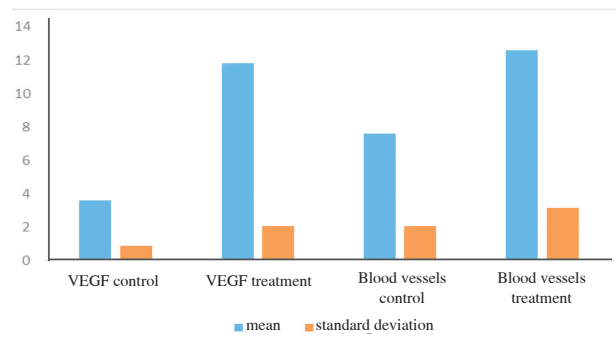


Figure 3. The mean and standard deviation of VEGF expressions and new blood vessels in the control and treatment groups.

With regard to normality test results, a Kolmogorov Smirnov test produced a p value of 0.634 ($p > 0.05$) for VEGF expressions and a p value of 704 ($p > 0.05$) for the new blood vessels. These results indicated the data to be normally distributed (Table 1). The mean of VEGF expressions in the treatment group was also confirmed to be higher than that in the control group. There was a significant difference in VEGF expressions between the control and

treatment groups with a p value of 0.000 ($p < 0.05$). The mean number of new blood vessels in the treatment group was also higher than that in the control group. Similarly, there was a significant difference in the number of new blood vessels between the control group and the treatment group with a p value of 0.018 ($p < 0.05$) (Table 2).

DISCUSSION

Bone tissue engineering innovation has focused on biomaterial applications, including scaffolds, being developed. Sponge-shaped scaffolds with a porous structure are suitable for cell attachment, cell proliferation, cell differentiation and specific tissue formation.¹² In this research, amniotic and gelatin membrane biomaterials were made of sponge with the objective of their being more easily applied to the post-extraction socket and of reducing post-extraction bleeding.

Amniotic membranes have anti-inflammatory, anti-bacterial, anti-fibrosis and anti-scarring properties with low immunogenicity, re-epithelialization effects, several growth factors, SLPI, collagen, fibronectin, nidogen, proteoglycans, hyaluronan, and laminin.^{7-9,13} The availability of almost infinite amniotic membranes and the process of obtaining them easily and cheaply for therapeutic purposes cause them to be regarded as having greater potential as biomaterials.¹⁴ Various growth factors are also found in amniotic membranes, including: EGF, KGF, HGF, FGF, TGF- α and TGF- β .⁹

The results of this research showed that there was a significant difference in VEGF expressions between the control and treatment groups. VEGF expressions are generally influenced by stimulation of the host, such as estrogen, nitric oxide (NO), various growth factors (bFGF, PDGF, TNF- α , TGF- β , EGF, and IGF1), as well as inflammatory cytokines (IL-6).^{15,16} In the early phase, post-extraction inflammation will occur, in which lactate levels increase and oxygen concentration is between 0-10 mmHg. The increased activity of inflammatory cells then leads to a hypoxic atmosphere and elevated lactate levels.

The latter, together with oxygen concentration at 0-10 mmHg, will attract young macrophages and fibroblasts migrated to the extraction sockets. Thereafter, macrophages in the wound will elaborate growth factors, known as macrophage-dependent angiogenic factors, resulting in chemotactic for endothelial cells. Endothelial cells then lead to the injury, and then release VEGF playing a role in angiogenesis and vasculogenesis.⁵

The results of this research also showed that the average number of new blood vessels in the treatment group was statistically higher than that in the control group. In other words, there was a significant difference in the number of new blood vessels between the control group and the treatment group. The results also revealed that the amniotic membrane was able to increase the number of new blood vessels since it contains FGF and TGF- β , two of the angiogenic factors.⁹ Angiogenic factors comprising VEGF, FGF, TNF- α , TGF- β , and PDGF bind to endothelial cell receptors around the site of the old blood vessels before activating, as well as generating, signals sent to the nucleus to produce protease enzymes which play an important role in the degradation of the extracellular matrix.⁵ Migration of endothelial cells then occurs to strengthen the branching structures of blood vessels, followed by the formation of new ones. Therefore, amniotic membranes can increase the number of new blood vessels.¹⁷

Several studies have shown that TGF- β plays a role in inhibiting the proliferation of endothelial cells in vitro and in vivo since TGF- β induces angiogenesis and stimulates VEGF expressions by attracting inflammatory cells.⁵ In infant rats, the administration of TGF- β at a dose of 1 μ g even can stimulate the occurrence of increased production of macrophages, fibroblasts, and collagen, as well as the formation of new capillaries.⁵ TGF- β may also regulate VEGF expressions through the apoptotic process of endothelial cells by activating TGF- β derived from vascular endothelial growth factor receptor-2 (VEGFR-2). The apoptotic process of endothelial cells is required for the formation of lumen within blood vessels.¹⁸

FGF, on the other hand, is known not only to stimulate endothelial cell proliferation in vitro (at concentrations of 1 to 10 ng / ml), but also to stimulate an in vivo angiogenic process leading to new blood vessel growth during the wound healing process by increasing the reendothelialization process in damaged blood vessels.⁵ Growth factors affecting VEGF expressions and existing in the amniotic membrane are FGF and TGF- β . Finally, it can be concluded that amnion sponge can increase the expressions of VEGF and the number of new blood vessels in the post-tooth extraction wounds of Wistar rats.

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