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Research Report

The pore size of chitosan-*Aloe vera* scaffold and its effect on VEGF expressions and woven alveolar bone healing of tooth extraction of *Cavia cobaya*

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ABSTRACT

Background: *Pore size of scaffolds affects cellular activity, stimulates angiogenetic factors of vascular endothelial growth factor (VEGF), synthesises new blood vessels to regulate migration and proliferation, and accelerates alveolar bone healing of tooth extraction.* ***Purpose:*** *This study aims to analyse the pore size of chitosan-Aloe vera scaffold and its effects on VEGF expression and woven alveolar bone healing of tooth extraction of Cavia cobaya.* ***Methods:*** *36 male Cavia cobaya, aged 3-3.5 months were divided into six groups: negative control groups (without scaffold), positive control groups (chitosan scaffold), and treatment groups (chitosan-Aloe vera scaffold) on 7- and 14-day observations. Histopathological examination was performed to account the woven alveolar bone areas, and immunohistochemical examination was conducted to examine VEGF expressions on endothelial cells. Data was analysed using a one-way analysis of variance (ANOVA) and least significant difference (LSD) test ($p<0.05$). Scaffold pore size examination was performed with scanning electron microscope (SEM) with 250x and 500x magnification.* ***Results:*** *Chitosan-Aloe vera scaffold was found to have open pore interconnectivity, the largest pore size was 138.9 μm, while the smallest was 110.5 μm and average pore size was 134.85 μm. The highest expression of VEGF was observed in the treatment group on days 7 (11.5 ±1.39) and 14 (15.28±1.78), while the largest woven alveolar bone was observed in the treatment group on days 7(17.83±1.47) and 14 (37.67±3.65). Statistically, there was a significant difference between control groups and the treatment groups ($p=0.000$; $p<0.05$).* ***Conclusion:*** *Chitosan-Aloe vera scaffold has pore characteristics increasing VEGF expressions and woven alveolar bone areas.*

Keywords: *Aloe vera; chitosan; scaffold pore size; VEGF; woven alveolar bone*

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INTRODUCTION

Alveolar ridge bone resorption often occurs after tooth extraction. Vertical and horizontal dimensional changes occur during the first three months after tooth extraction.[1] Alveolar bone resorption will remain and even can cause more than 40 – 60% ridge volume loss during the first three years post tooth extraction.[2–4] The damage of the alveolar bone, unfortunately, can cause failure or the instability of dentures or dental implant placement.[4,5]

Ridge preservation and grafting materials can be used to prevent bone loss and to regenerate alveolar bones. Although grafting materials do not completely prevent bone loss, it could reduce the severity of the loss.[1,6] Grafting materials currently used are autograft, bovine xenograft, allografts and alloplast. However, the use of bovine grafts to the tooth socket still cannot bring satisfactory results. There are developments of a combination of natural and synthetic graft materials or polymers used to achieve alveolar bone resorbtion.[1,2] Actually, bone tissue engineering innovation has recently developed scaffold that can be absorbed by the body, such as chitosan polymers material, in order to accelerate the replacement of damaged tissue as well as to proliferate, differentiate and maintain tissue function.[7] The application of chitosan to the tooth extraction socket of *Rattus norvegicus* can increase the number of osteoblasts,

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fibroblasts and type I collagen on 7 and 14 days of observation.[8] One percent of chitosan gel is also known to be able to increase bone morphogenetic protein-2 (BMP-2) expressions of *Rattus norvegicus* during bone formation after tooth extraction on 7, 14 and 21 days.[9]

Aloe vera is a natural plant that can be used as a biogenic stimulator to stimulate and accelerate alveolar bone regeneration. *Aloe vera* has active compounds that play a role in the bone healing process. It is a compound protein named alloktin that is synergistic with the other components like amino acids, enzymes, alkaloids, flavonoids, saponins, collagen, vitamins, calcium, potassium and polysaccharides mannan.[10–12] Hence, in the previous study, the use of *Aloe vera* scaffold containing acemannan increased bone marrow stromal cells (BMSCs), VEGF, BMP-2 proliferations, alkaline phosphatase (ALP) activity, bone sialoprotein, mineralisation and osteopontin expressions on bone healing of tooth extraction. *Aloe vera* can be considered as a natural candidate for bone regeneration.[13]

Scaffold made of the combination of chitosan and *Aloe vera* is assumed to have a synergistic effect on tooth extraction sockets to regenerate the alveolar bone and prevent alveolar bone resorption. Chitosan has osteoconductivity that can support the attachment of bone-forming cells. *Aloe vera* was also shown to have high osteoinductivity and osteogenity that can stimulate the differentiation of osteoprogenitor cells into osteoblast cells. Thus, it also can trigger new bone formation and bone regeneration.[10,14]

The characteristic porosity and pore size of scaffold are known to be able to affect cellular activities including stimulating new cell growth and cell adhesion as well as supporting cell proliferation and angiogenetic factors so that it will accelerate the bone healing process. VEGF is the most dominant growth factor considered as an angiogenetic factor released by endothelial cells, which can synthesise new blood vessels to regulate the migration, proliferation and differentiation processes of endothelial cells and the formation of new bone.[15,16] Furthermore, this study aims to analyse the pore size of chitosan-*Aloe vera* scaffold and its effects on VEGF expression and woven alveolar bone healing of tooth extraction of *Cavia cobaya* (*C. cobaya*) on 7- and 14-day observations.

MATERIALS AND METHODS

This study was an experimental study with a randomised post-test only control group design. The chitosan powder used in this study had a deacetylation degree of >75–85% and a molecular weight of 50,000–190,000 Da (Sigma, Product number: 448869, Lot number: MKBH7256V). 1% chitosan gel (w/p) was made by dissolving 1 gram of chitosan powder in 100 mL of 2% acetic acid (CH3COOH). It was stirred using a magnetic stirrer, neutralised with NaOH solution, centrifuged at a speed of 2000 rpm for 30 minutes and filtered with filter paper. *Aloe vera* extract gel was made by the maceration method. *Aloe vera* was cleaned, and its thorns were removed. *Aloe vera* was blended until smooth, dried with a freeze dry machine, dissolved with 70% ethanol for 48 hours and stirred for 30 minutes with a magnetic stirrer. The maceration results were filtered with Whatman grade 1 filter paper and accommodated with Erlenmeyer. The filtrate was evaporated with a vacuum rotary evaporator and dissolved using 3.5% sodium carboxymethyl cellulose (Na-CMC). Subsequently, chitosan-*Aloe vera* scaffold was made by mixing the chitosan gel and the ethanol extract of Aloe vera gel in a ratio of 1:1. The combination of chitosan and *Aloe vera* gel was put into the scaffold mould after it was put in a freezer at a temperature of -80° C for 24 hours. Afterwards, freeze drying was carried out at a temperature of 95°–103° C for 72 hours.[17] The scaffold was removed from the mould and sterilised with a UV clean bench steriliser. Scaffold pore size examination was performed with a scanning electron microscope (SEM) tool (JCM-5700, JEOL, Tokyo, Japan) with 250x and 500x magnification.

Ethical approval for this research was obtained from the ethical committee of Airlangga University, Faculty of Dental Medicine with number 012 / HRECC.FODM / III / 2018. In this study, the experimental animals used were 36 male *C. cobaya* aged 3–3.5 months and weighed 300–375 grams. The sample size was determined by using Lameshow's minimum sample size formula. The sample was selected blind-randomly into control and treatment groups, which were divided into six groups. Each group consisted of 6 *C. cobaya* divided into six groups: on day 7: negative control groups (without scaffold administration), positive control groups (chitosan scaffold administration), and treatment groups (chitosan-*Aloe vera* scaffold administration). On day 14: negative control groups (without scaffold administration), positive control groups (chitosan scaffold administration), and treatment groups (chitosan-*Aloe vera* scaffold administration). The left mandibular incisor of *C. cobaya* was extracted. The tooth socket was irrigated with sterile aquadest water. The scaffold was applicated in the tooth socket to the apical end of the tooth and sutured with non resorbable sutures. *C. cobaya* from each group were sacrificed after 7 and 14 days. The mandibular bone in the interdental region of the mandibular incisors was cut and soaked in a fixation solution using 10% formalin buffer. A decalcification process was carried out with 10% of ethylenediaminetetraacetic acid (EDTA) (Onemed Dental, Medika Industri, Indonesia) for 4 weeks. Subsequently, paraffin blocks were made and cut with microtome in a buccolingual plane parallel to the tooth vertical axis into sections of 4-micro thickness. Histopathological examination was conducted with haematoxylin eosin (HE) staining (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) to account the woven alveolar bone areas using Image Raster software version 3 (developed by PT MICONOS, Yogyakarta, Indonesia). Light microscope (Nikon E100, Tokyo, Japan) on 100x magnification on five different fields of view by two observers was performed.

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The immunohistochemical examination was conducted using a 3.3'-diaminobenzidine stain kit (DAB) (Sigma Aldrich, Merck KGaA, Darmstadt, Germany). The antibody monoclonal VEGF (ab38909, abcam, United Kingdom) was used to measure the VEGF expressions in the apical third of teeth, which were viewed using a light microscope (Nikon E100, Tokyo, Japan) in 400x magnification on five different fields of view by two observers.

The data was analysed by Statistical Package for the Social Sciences 21.0 software (SPSS for Windows, Chicago, USA). Data analysis was performed using the normality test with the Shapiro Wilk test. A homogeneous variation test was conducted to find out data variation in the groups with levene's test ($p>0.05$) continued with one-way ANOVA and multiple comparison LSD test ($p<0.05$) to determine the different pairs of the groups.

RESULTS

The results of pore size examination of chitosan-*Aloe vera* scaffold using SEM tool with 250x and 500x magnifications showed the largest scaffold pore size was 138.9 µm, the smallest scaffold pore size was 110.5 µm, and the average scaffold pore size was 134.85 µm. Open pore interconnectivity of chitosan-*Aloe vera* scaffold was found. There was interconnection between pores on the scaffold. The pore size and connectivity of scaffold can be seen in Figure 1.

Based on Figure 2, the VEGF expression of endothelial cells in the treatment group with chitosan-*Aloe vera* on 7 and 14 days increased more than negative control groups and positive control groups with chitosan scaffold. The VEGF expression in the negative control group (without scaffold administration), positive control group with chitosan scaffold and treatment group with chitosan-*Aloe vera* scaffold in 7 days can be seen in Figures 2a, c, e. The VEGF expression in negative control group (without scaffold administration), positive control group with chitosan scaffold and treatment group with chitosan-*Aloe vera* scaffold in 14 days can be seen in Figures 2b, d, f.

The width of woven alveolar bone in the treatment group with chitosan-*Aloe vera* in 7 and 14 days increased more, as pointed by red arrows, than the negative control group and positive control group with chitosan scaffold. Figure 3a, c, e shows the width of woven alveolar bone in the negative control group (without scaffold administration), positive control group with chitosan scaffold and treatment group with chitosan-*Aloe vera* scaffold in 7 days. Figure 3b, d, f shows the width of woven alveolar bone in the negative control group (without scaffold administration), positive control group with chitosan scaffold and treatment group with chitosan-*Aloe vera* scaffold in 14 days

Based on Table 1 and Figure 4, the results of the analysis showed that treatment groups with chitosan-*Aloe vera* scaffold could significantly increase the VEGF expressions and the width of woven alveolar bone areas in 7 and 14 days compared with the negative control group and positive control group with chitosan scaffold ($p<0.05$). The highest VEGF expression and the largest woven alveolar bone were found in the treatment groups with chitosan-*Aloe vera* scaffold. The VEGF expression and width of woven alveolar bone in the positive control group with chitosan scaffold could increase more than negative control groups without scaffold administration.

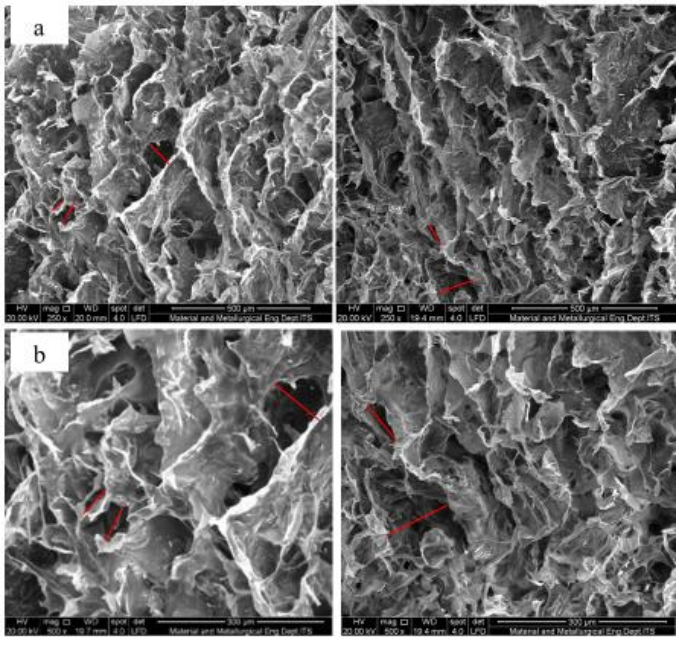

Figure 1. The pore size of the chitosan-*Aloe vera* scaffold using SEM tool with the magnifications of 250x (a) and 500x (b): the red lines shows the pore size of scaffold.

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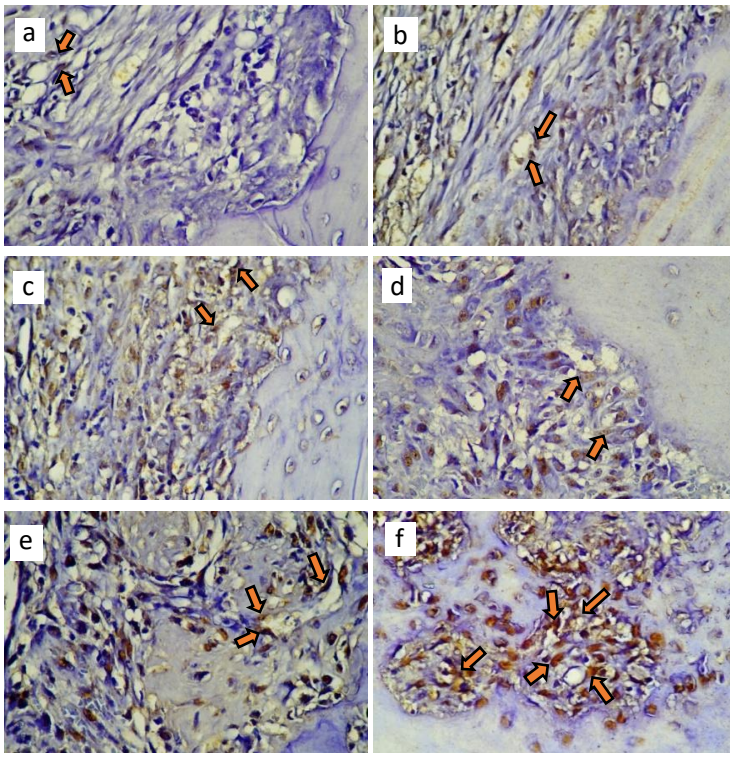

Figure 2. The VEGF expression on endothelial cells. (a) K(-) group on day 7; (b) K(-)group on day 14; (c) K(+) groups with chitosan scaffold on day 7; (d) K(+) groups with chitosan scaffold on day 14; (e) The treatment group with chitosan-*Aloevera* scaffold on day 7; (f) The treatment group with chitosan-*Aloe vera* scaffold on day 14, with 400x magnification; the orange arrow shows VEGF expression on endothelial cells.

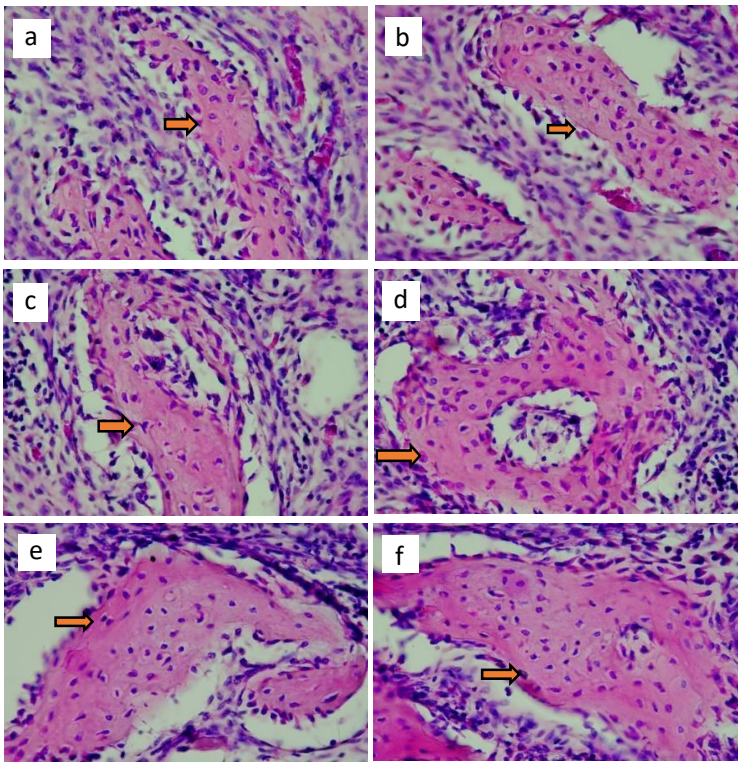

Figure 3. The woven alveolar bone areas. (a) K(-) group on day 7; (b) K(-)group on day 14; (c) K(+) groups with chitosan scaffold on day 7; (d) K(+) groups with chitosan scaffold on day 14; (e) The treatment group with chitosan-*Aloe vera* scaffold on day 7; (f) The treatment group with chitosan-*Aloe vera* scaffold on day 14, with 100x magnification; the orange arrow shows the width of woven alveolar bone areas.

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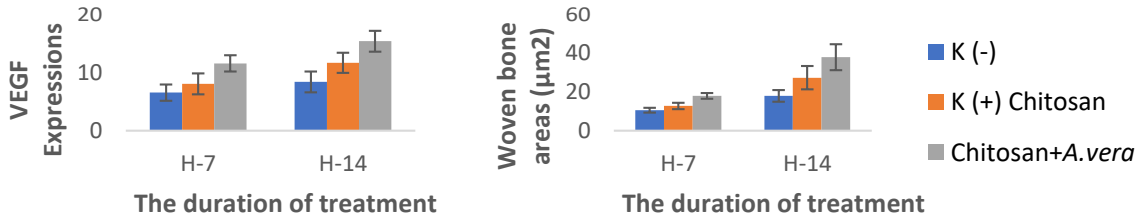

Figure 4. Diagram of VEGF expressions and woven bone area in K(-) groups, K(+) groups with chitosan scaffold and treatment groups with chitosan-*Aloe vera* scaffold on 7 and 14 days.

Table 1. The mean and standard deviation of VEGF expressions and woven bone areas in all groups

Groups	N	VEGF Expression (cells/LP)	Woven Bone Areas (μm^2)	P
		$\bar{x}$ ±SD	x ±SD	
K (-) on day 7	6	6.50 ±1.64[a]	10.50±1.23[a]	0.000*
K (-) on day 14	6	8.33±1.75[a]	17.83 ±2.99[c]	
K (+) chitosan on day 7	6	8.00±1.79[a]	12.67±1.63[b]	
K (+) chitosan on day 14	6	11.60±1.72[b]	27.17±3.98[d]	
Chitosan+*A.vera* on day 7	6	11.50±1.39[b]	17.83±1.47[c]	
Chitosan+*A.vera* on day 14	6	15.28±1.78[c]	37.67±3.65[e]	

Note: * significant at α=0.05 (one-way ANOVA)
[abc] different superscripts show that there were differences between groups (multiple LSD comparisons)

DISCUSSION

In the development of tissue engineering, the use of chitosan scaffold in medical applications has been mostly modified by many crosslinks with other ingredients such as collagen, gelatin, hydroxyapatite or growth factors to increase osteoinduction and osteointegration resulting in the acceleration of the bone healing process. The single use of chitosan as scaffold has inadequate pore size, poor porosity and close interconnectivity to facilitate the transportation of nutrients, growth factors and blood vessels.[7,18,19]

Scaffold made of the combination of chitosan and *Aloe vera*, based on the SEM test results, has a mean pore size of 134.85 µm. The chitosan-*Aloe vera* scaffold has a good pore interconnectivity or open pore interconnectivity. The recommended minimum pore size for scaffold is 100 µm, which enables the scaffold not only to provide a good and suitable microenvironment for the proliferation of osteoblasts and mesenchymal stem cells as well as the attachment and migration of cells, but also to be capable of nutrient diffusion. Open pore interconnectivity can also increase tissue vascularisation and oxygenation, which support the bone healing process. Pore size and pore interconnectivity of scaffold affect cellular activity, stimulate angiogenetic released by endothelial cells and also synthesise new blood vessels to regulate migration, proliferation and new bone formation.[20,21] In our study, the use of chitosan-*Aloe vera* scaffold could increase VEGF expressions as well as woven alveolar bone areas in the 7- and 14-day observation compared to the use of chitosan scaffold.

Moreover, the alveolar bone healing process of tooth extraction actually begins with a haemostasis phase, which activates platelets and blood clotting factors to form a blood clot that fills the socket. The cytoplasm of platelets contains α granules containing growth factors such as platelet derived growth factor (PDGF) and transforming growth factor-β (TGF-β). These molecules can activate and attract polymorphonuclear cells (PMNs), macrophages and endothelial cells to the socket. Macrophages are the main cells that play an important role in the healing process involving phagocytosis and secretion of cytokines and growth factors that modulate the bone healing process.[22,23] In the final inflammatory phase, macrophage cells begin to stimulate the increase of induced growth factors such as PDGF, fibroblast growth factor (FGF), VEGF, TGF-β and transforming growth factor-α (TGF-α).[23,24] VEGF is the most dominant angiogenetic factor released by endothelial cells to synthesise new blood vessels to regulate the migration, proliferation and differentiation processes.[15,16] Hence, the VEGF expressions in the treatment groups with the administration of the chitosan-*Aloe vera* scaffold in this study tended to increase. The increasing of VEGF expressions in those treatment groups after day 7 was not even significantly different from that in the groups with the administration of the chitosan scaffold on day 14. It may be caused by the inflammatory phase still ongoing before the 7th day, so a time lag is needed to lead to the proliferation phase. As a result, the release of growth factors that induce VEGF has not been maximised yet.[22]

Differentiated osteoblasts on the apical third region of the tooth socket form a bone matrix, and immature or woven alveolar bones begin from the apical region of the socket to the lateral wall of the socket on day 7 and then extend to the centre of the socket leading to trabecular bones. Along with the healing process of the alveolar bone after the complete

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tooth extraction, the area of woven alveolar bone will be greater.[25] This can also be seen in the results of this study on the 7th day when the formation of woven alveolar bone had occurred in both the control groups and the treatment groups. The width of woven alveolar bone areas had even been getting greater in all groups from day 7 to day 14.

Furthermore, angiogenesis is a key component in the bone healing process. During the bone healing process the formation of new blood vessels is also needed in metabolic callus regeneration for the supply of nutrients, oxygen, growth factors, cytokines, osteoblast precursors and osteoclasts.[16] In the proliferation phase, for instance, angiogenesis plays an important role during the migration of endothelial cells into proliferating new tissue. In a normal alveolar bone healing process post tooth extraction the proliferation phase is started with the onset of hypoxic conditions causing an increase in intracellular concentration of the active form of a gene regulating protein called hypoxia-inducible factor 1 (HIF-1). This condition triggers endothelial cells and macrophages to release angiogenetic factors in response to inflammation and increased HIF-1. Subsequently, endothelial cells and macrophages will secrete angiogenetic factors such as basic fibroblast growth factor (bFGF or FGF-2) and acid FGF (aFGF or FGF-1), PDGF, VEGF, and TGF-β. bFGF then will produce mature endothelial cells and synthesise new blood vessels. Afterwards, cell surface receptors will bind to VEGF and FGF which are activated by kinase receptors so that they can regulate the migration, proliferation and differentiation processes of endothelial cells.[15,16] Thus, in the control groups of this study, the mean number of VEGF expressions increased from day 7 to day 14 although there was no significant difference. This means that in post tooth extraction conditions the bone healing process without scaffold administration in tooth sockets that have tissue damage is a hypoxic condition triggering bFGF and VEGF secreted by endothelial cells. In contrast, the number of VEGF expressions in the groups with the administration of the chitosan scaffold and that in the groups with the administration of chitosan-*Aloe vera* scaffold increased after day 7, and the increasing of VEGF expressions in those groups was even significantly different between those on 7 and 14 days. This indicates that the process of angiogenesis in the treatment groups supports the process of alveolar mineralisation.

Chitosan as a natural biopolymer containing glycosaminoglycans is known not only to have unique properties, biocompatible and biodegradable characteristics but also to be able to stimulate the release of important growth factors in bone healing such as FGF, PDGF, TGF-β1, VEGF, BMP-2 and collagen type 1.[8,9,26] Hence, in this study the VEGF expressions and the width of woven alveolar bone areas on 7 and 14 days in the groups with the administration of the chitosan scaffold and those in the groups with the administration of chitosan-*Aloe vera* scaffold were increasing and significantly different from those in the control groups. The results of this study also revealed that VEGF expressions and the width of woven alveolar bone areas on 7 and 14 days in the groups with the administration of chitosan-*Aloe vera* scaffold were significantly different from those in the control groups and the groups with the administration of the chitosan scaffold. The highest average and increase of VEGF expressions and the width of woven alveolar bone areas on 7 and 14 days were found in the groups with the administration of chitosan-*Aloe vera* scaffold compared to the other groups.

The increased VEGF expressions in the use of *Aloe vera* is known to be through the phosphatidylinositol 3-kinase (PI3K/Akt), extracellular-signal-regulated kinase (ERK 1/2) and endothelial nitric oxide synthase / nitric oxide (eNOS/NO) pathways.[27,28] HIF-1 alpha binds to the hypoxic response element in the VEGF gene promoter which stimulates transcription. VEGF binds to two VEGF receptors, VEGFR-1 / Flt (Fms-like tyrosine kinase) and VEGFR-2/KDR. VEGFR-2 activation is linked to mechanisms that depend on the formation of multi-protein complexes including VEGFR-2, PI3K, as well as VE-cadherin and β-catenin proteins. VEGF binds to serine receptors on endothelial cells then initiates VEGFR-2 autophosphorylation followed by activation of angiogenesis enzymes such as MAPK and Akt / kinase B protein (PKB) to induce cell migration. ERK 1/2 pathway plays an important role in the growth and differentiation mechanisms of endothelial cells during the process of angiogenesis in wound healing.[16,27] Subsequently, through the ERK 1/2 pathway and the c-Jun N-Terminal Kinase (JNK) pathway, the chitosan-*Aloe vera* scaffold will activate macrophages with M2 modulation more dominant than M1. In M2 modulation, macrophages will activate M2 which stimulates anti-inflammatory cytokines, IL-2 and IL-10. In addition, macrophages also induce cell migration and proliferation by activating activator protein-1 (AP-1), which then activates FGF, VEGF and BMP-2 playing a role in stimulating osteoblast formation.[27,29] Bonding components of the lectin protein or Aloktin with *Aloe vera* polysaccharides will activate the complement system and increase coagulation to prevent loss of blood clots in bone healing.[30,31] The interactions of the protein components, such as lectin, polysaccharides, anthraquinone and beta-sitosterol are then identified as angiogenetic factors in the healing process since they stimulate human umbilical vein endothelial cells (HUVEC).[27,32] Polysaccharides and flavonoids contained in *Aloe vera* can also increase angiogenic factors in BMSCs.[33]

The administration of *Aloe vera* to tooth sockets and alveolar bone defects can increase the expression of runt-related transcription factor 2 (Runx2) that plays a role in inducing pre-osteoblast differentiation into mature osteoblasts. As osteoblasts increase, the expression of osteoprotegerin (OPG) released by osteoblasts increases as does ALP activity. As a result, osteoclastogenesis can be prevented through receptor activation of nuclear factor kappa B ligand (RANKL)/receptor activator of nuclear

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factor kappa B (RANK)/OPG system signals. Runx2 then induces osteoblasts to secrete osteopontin, osteocalcin and type 1 collagen, which influence the mineralisation and bone healing processes.[32–34] Therefore, it can be concluded that chitosan-*Aloe vera* scaffold has pore characteristics increasing VEGF expressions and woven alveolar bone areas on alveolar bone healing of tooth extraction on *C. cobaya*.

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