Surabaya, 10 Juni 2020

Nomor : 97/EXT-DENTJ/VI/2020
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Departemen Ilmu Material Kedokteran Gigi
Fakultas Kedokteran Gigi Universitas Hang Tuah
Jl. Arif Rachman Hakim 150 Surabaya 60111

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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
Authors: Sularsih

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Saka Winias, drg., M.Kes., Sp.PM
NIP. 199005152014042000

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Best regards
Dr. Sularsih drg,MKes

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Dental Journal
Majalah Kedokteran Gigi

Surabaya, 21 Juli 2020

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Kepada Yth.
Dr. Sularsih, drg., M. Kes
Departemen Ilmu Material Kedokteran Gigi
Fakultas Kedokteran Gigi Universitas Hang Tuah
Jl. Arif Rachman Hakim 150 Surabaya 60111

Kami beritahukan bahwa naskah sejawat dengan judul :

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*
Author: Sularsih

telah **diterima** dan naskah tersebut akan diterbitkan pada Dental Journal (Majalah Kedokteran Gigi) volume 53 nomor 3 – September 2020.

Naskah selanjutnya akan melalui proses copyediting, proofreading, layouting dan publishing.

Demikian surat keterangan ini kami buat mohon diterima dan digunakan seperlunya, atas perhatiannya kami sampaikan terima kasih.

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Dental Journal
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Saka Winias, drg., M.Kes., Sp.PM
NIP. 199005152014042000

FORMAT PENILAIAN NASKAH DENTAL JOURNAL HASIL PENELITIAN (untuk Penyunting Ahli)

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Yogyakarta, 30/6/2020

Penyunting Ahli,

Research Report

The Pore size of Chitosan-Aloevera Scaffold and its effect on VEGF expressions and woven alveolar bone healing of tooth extraction of Caviacobaya

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ABSTRACT

Background: Microporosityand pore size of scaffold affect cellular activity, stimulate angiogenetic factors of Vascular Endothel Growth Factor (VEGF) released by endothelial cells, and also synthesize new blood vessels to regulate the migration, proliferation, and new bone formation.***Purpose:*** This study aims to analyze the pore size of chitosan-Aloe vera scaffold and its effects on VEGF expression and woven alveolar bone healing of tooth extraction of Caviacobaya. ***Methods:*** thirty-six male Caviacobaya, aged 3 - 3.5 months were divided into three groups, with each group consisting of 12 Caviacobaya: Group I: negative control groups (without scaffold administration), group II: positive control groups (with chitosan scaffold administration), and group III: treatment groups (with chitosan-Aloe vera scaffold administration). The scaffold was applied to the sockets. SixCaviacobaya from each group were sacrificed after 7 and 14 days.The mandibular bone was cut. Histopathological examination was performed to account the woven alveolar bone areasand immunohistochemical examination was conducted to analyze of VEGF expressions. ***Results:***The largest pore size of chitosan-Aloe vera scaffold was 139.9 µm, while the smallestone was 110.5 µm. The average pore size was 124.85 µm. It was found open pore interconnectivity in the chitosan-Aloe vera scaffold. The use of Chitosan-Aloe verascaffold could increase VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days observation. Statistically,there was a significant difference between control groups and the treatment groups with chitosan- Aloe verascaffold ($p < 0.05$). ***Conclusion:*** Chitosan-Aloe vera scaffold has pore characteristics that can allow good vascularization and also accelerate alveolar bone healing processof tooth extraction in Caviacobaya through increasing VEGF expressions and the width of woven alveolar bone areas.

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

INTRODUCTION

Based on the Health Ministry's Research and Health Agency survey report in 2018, the average M-T (missing teeth) index in Indonesia was 2.5. This means that the average number of tooth lost in Indonesia was 250 teeth per 100 people indicating on average every person in Indonesia lost 3 teeth.[1] Besides, it is also known that the case of tooth loss is due to the high prevalence of periodontal disease in Indonesia, which ranks second in dental health problemsin Indonesia.[2]For instance, alveolar bone resorption often occurs after tooth extraction.[3] Alveolar bone resorption then will keep staying, and even can cause more than

40% - 60% of ridge volume lose during the first 3 years post tooth extraction.[3,4]The damage of alveolar bone, unfortunatelycan cause failure or the instability of denture or dental implantplacement.[4,5]

One of the periodontal treatments to preserve tooth sockets to regenerate alveolar bone and prevent alveolar bone resorption is by using bone graft material on bone defects.[6]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 norvegicuscan increase the number of osteoblast cells, fibroblast cells, and type I collagen on the 7th and 14th days of observation.[8] Chitosan gel 1% is also known to be able to increase Bone Morphogenetic Protein-2 (BMP-2) expressionsof Rattus norvegicus during bone formation after tooth extraction on days 7,14, and 21.[9]

Aloe verais 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 healing process. Its compounds protein (alloktin), amino acids, enzymes, alkaloids, flavonoids, saponins, collagen, vitamins, calcium, potassium, and polysaccharides mannan.[10,11,12]Hence, in the previous study, the use of Aloe verascaffold containing acemannanwas increase BMSCs, VEGF, and BMP-2proliferations, ALP activity, bone sialoprotein, mineralization, and osteopontin expressions on bone healing of tooth extraction. Aloe veracan be considered as a natural candidate for bone regeneration.[13]

Therefore, scaffold made of the combination of chitosan and Aloe verais assumed to have a synergistic effect on tooth extraction sockets to regenerate alveolar bone and prevent alveolar bone resorption. Chitosan is osteoconductionthat can support the attachment of bone-forming cells.Meanwhile, Aloe vera is osteoinduction and osteogenesisthat can stimulate the differentiation of osteoprogenitor cells into osteoblast cells and also can trigger new bone formation and bone regeneration.

Microporositystructure and pore size of scaffold are known to be able to affect cellular activities, including stimulating new cell growth, cell adhesion as well as supporting cell proliferation and angiogenetic factor so that it will accelerate bone healing process. VEGF is the most dominant growth factor considered as an angiogenetic factor released by endothelial cells,which can synthesize new blood vessels to regulate the migration, proliferation, and differentiation processes of endothelial cells and the formationof new bone.[15,16] Thus, this study aims to analyze the pore size of chitosan-Aloe vera scaffold and its

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effects on VEGF expression and woven alveolar bone healing of tooth extraction of Caviacobayaon the 7^{th} and 14^{th} days of observation.

MATERIALS AND METHODS

Chitosan powder used in this studywas chitosan powder with a deacetylation degree of > 75-85%and a molecular weight of 50,000-190,000 Da (Sigma, Product number: 448869, Lot number: MKBH7256V). Chitosan gel 1% (w/p) was made by dissolving 1 gram of chitosan powder in 100 mL of acetic acid (CH3COOH) at a concentration of 2%. After that, it was stirred using a magnetic stirrer, neutralized with NaOH solution, centrifuged at a speed of 2000 rpm for 30 minutes,and then filtered with filter paper. Aloe vera extract gel was made by maceration method.Aloe verawas cleaned, and its thorns were removed. Its gel was taken. The Aloe vera gel was blended until smooth, dried with a Freeze dry ~~device~~, dissolved with 70% ethanol, ~~and~~ then stirred for 30 minutes with a magnetic stirrer. The maceration results were filtered with a buctner funnel coated with filter paper and accommodated with erlenmeyer. The ~~filtered~~ filtrate was evaporated with a vacuum rotary evaporator, ~~and~~ then dissolved using 3.5% Sodium carboxymethyle 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 then was put into the scaffold mold after it was put in freezer at a temperature of -80 degrees for 24 hours.Afterwards, freeze drying was carried out at a temperature of 95-103 degrees for 72 hours. The scaffold then was removed from the mold and sterilized with a UV clean bench sterilizer. Scaffold pore size examination was performed with a Scanning Electron Microscope (SEM) ~~tool~~ (JCM-5700, JEOL, Tokyo, Japan) with 250x and 500x magnification.

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This study was an experimental research with randomized post test only control group design. Ethical Approval for this research was obtained from the Ethical committee of Airlangga University Faculty of Dentistry no 012 / HRECC.FODM / III / 2018. In this study, experimental animals used were thirty-six male Caviacobaya,aged 3 - 3.5 monthsand weighed 300- 375 grams. Those Caviacobayaanimals were divided into three groups, with each group consisting of 12 Caviacobaya: Group I: negative control groups (without scaffold administration), group II: positive control groups (with chitosan scaffold administration), and group III: treatment groups (with chitosan-Aloe vera scaffold administration). SixCaviacobaya from each group were sacrificed after 7 and 14 days.Tooth extraction was performed on the left mandibular incisor. The tooth socket then was irrigated with sterile aquadest liquid. After that, the sterilized scaffold was applicated in the tooth socket to the apical end of the tooth, and sutured with non resorbable sutures. Those animals then were decaputated on days 7 and 14 after the treatment. Afterwards, the jaw bone in the interdental region of the mandibular incisors was cut and inserted in a fixation solution using formalin buffer10%. Decalcification process then was carried outwith EDTA for 4 weeks. Subsequently, paraffin blocks were made. Histopathological examination then was conductedwith hematoxylin eosin (HE) staining to account the width of woven alveolar bone areas using Image Raster 3 software.The immunohistochemical examination was performed with DAB chromogen kit on the Monoclonal Anti-Caviacobaya Vascular Endothelial Growth Factor Antibody to measure the VEGF expressions in the apical third of teeth.

Data analysis was performed using normality test with Shapiro Wilk test. Homogeneous variation test then was conducted to find out data variation inthe groups with Levene's test at a significance of 5%. If the data was normally distributed and had homogeneous data variations, a statistical analysis of variance analysis would be carried out and continued with a multiple comparison LSD test ($p < 0.05$). But, if the data were not normally distributed, the Kruskal-Walis non-parametric test was performed, and continued with Wilcoxon-Mann Whitney analysis to determine the different pairs of the groups.

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RESULTS

SEM Test Results

The results of the SEM test on the chitosan-Aloe vera scaffold with 250x and 500x magnifications showed the largest scaffold pore size of 139.9 μm, the smallest scaffold pore size of 110.5 μm, and the average pore size of 124.85 μm. It was found a good pore interconnection or open pore interconnectivity of chitosan-Aloe vera scaffold(Figure 1).

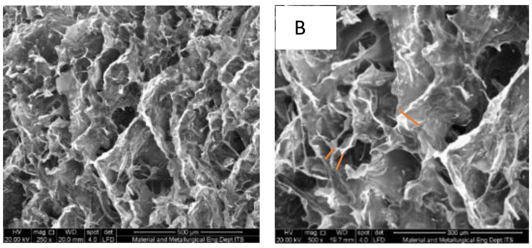

Figure 1. SEM test results on the pore size of the chitosan-Aloe vera scaffold with the magnifications of 250x (a) and 500x (b)

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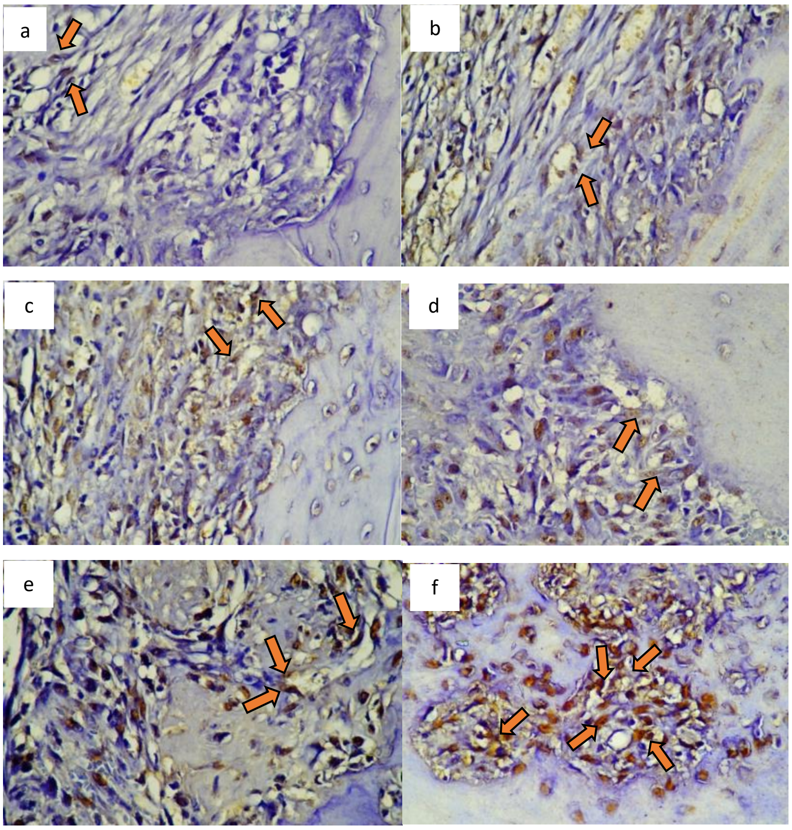
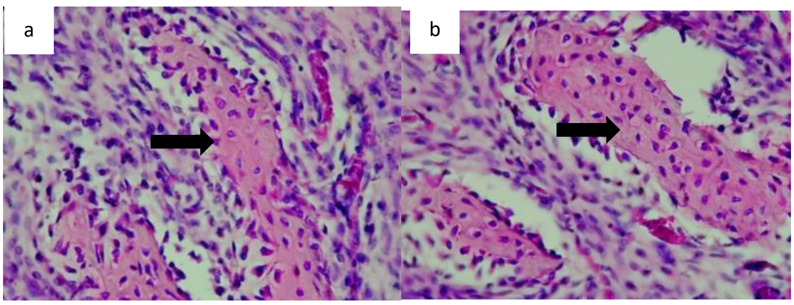


Figure 2. VEGF expressions on endothelial cells showing brown color marked with arrows. (a) The control group on day 7, (b) The control group on day 14, (c) The treatment group with chitosan scaffold on day 7, (d) The treatment group with chitosan scaffold on day 14, (e) The treatment group with chitosan-Aloe vera scaffold on day 7, (f) The treatment group with chitosan-Aloe vera scaffold on day 14, with 400x magnification



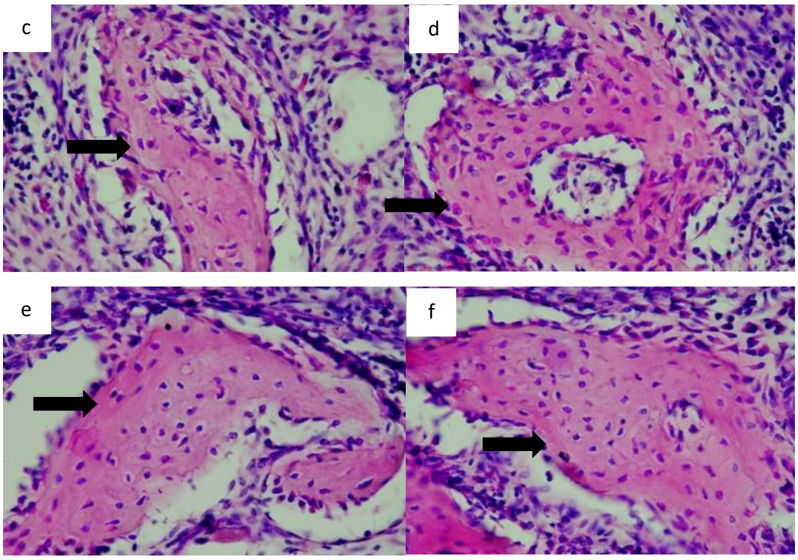


Figure 3. Thewoven alveolar bone areas. (A) The control group on day 7, (B) The control group on day 14, (C) The treatment group with chitosan scaffold on day 7, (D) The treatment group with chitosan scaffold on day 14, (E) The treatment group with chitosan-Aloe vera scaffold on day 7, (F) The treatment group with chitosan-Aloe vera scaffold on day 14, with 100x magnification

Table1. The mean and standard deviation (SD)of VEGF expressions in all groups

Groups	**N**	**VEGF Expressions (cells/LP)**				**P**
		$\bar{x}$	**SD**	**Min**	**Max**	
Control on day 7	6	6.50^a	1.64	4.0	8.0	0.000*
Control on day 14	6	8.33^a	1.75	6.0	11.0	
Chitosan on day 7	6	8.00^a	1.79	5.0	10.0	
Chitosan on day 14	6	11.60^b	1.72	8.3	13.0	
Chitosan+*A.vera* on day 7	6	11.50^b	1.39	10.0	14.0	
Chitosan+*A.vera* on day 14	6	15.28^c	1.78	13.7	18.0	

Note: * significant at α=0.05 (*OnewayAnova*)
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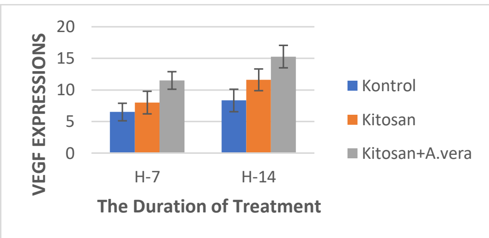

Figure 4. The Diagram ofVEGF expressions in the control groups, the treatment groups with chitosan scaffold, and the treatment groups with chitosan-Aloe vera scaffold on days 7 and 14

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Table 2. The mean andstandard deviation of Woven Bone Areas in all groups

Groups	n	Woven Bone Areas (µm²)					P
		$\bar{x}$	SD	Median	Min	Maks	
Control on day 7	6	10.50	1.23	11.0 [a]	9	12	0.000*
Control on day 14	6	17.83	2.99	17.5 [c]	13	21	
Chitosan on day 7	6	12.67	1.63	12.5 [b]	11	15	
Chitosan on day 14	6	27.17	5.98	26.0 [d]	21	38	
Chitosan+*A.vera* on day 7	6	17.83	1.47	18.0 [c]	15	19	
Chitosan+*A.vera* on day 14	6	37.67	6.65	35.5 [e]	32	49	

Note: * significant at α = 0.05 (Kruskal-Wallis test)
[abcde] Different superscripts show differences between groups (Mann-Whitney test)

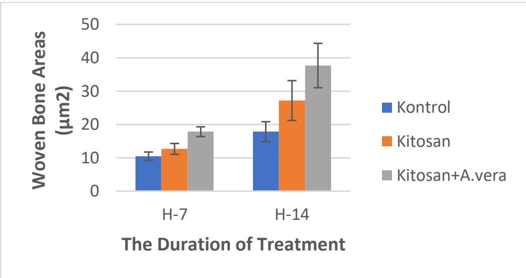

Figure 5. The Diagramof woven alveolarbone areas in the control groups, the treatment groups with chitosan scaffold, and the treatment groups with chitosan-Aloe vera scaffold on days 7 and 14

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The results of the analysis showed that the use of chitosan-Aloe vera scaffold could significantly increase theVEGF expressions and the width of woven alveolarbone areas on the 7th and 14th days compared with the control group and the group with application of chitosan scaffold (table 1 and 2).

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 bone healing process. The single use of chitosan as scaffold has unadequate pore size, poor porosity, and close interconnectivity to facilitate the transportation of nutrients, growth factors, and blood vessels.[7.17,18]

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Unlike the scaffold made of the single use of chitosan, scaffold made of the combination of chitosan and Aloe vera,based on the SEM test results,has a mean pore size of 124.85 µm. The chitosan-aloe vera scaffold has a good pore interconnectivity or open pore interconectivity. The recommended minimum pore size for scaffold is 100 µm,which enables the scaffold not only to provide a good micro or nich environment for the proliferation of osteoblastsand 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 vascularization 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 alsosynthesize new blood vessels to regulate the migration, proliferation, and new bone formation. [19,20]In our study, the use of chitosan-Aloe verascaffold could increase VEGF expressionsas well as the width of woven alveolar bone areas on the 7th and 14th day compared to the use of chitosan scaffold.

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Moreover, alveolar bone healing process of toothextraction actually begins with hemostasis 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 PDGF and TGF-β. These molecules can activate and attract PMNs, macrophages, and endothelial cells to thesocket. Macrophage cells 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.[21,22]In the final inflammatory phase, macrophage cells begins to stimulate increasing of induced growth factors as PDGF, FGF, VEGF, TGF-β, and TGF-α.[22,23] VEGF is the most dominant angiogenetic factors released by endothelial cells to synthesize 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 even was not 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 maximized yet.

Commented [HP15]: ?

Differentiated osteoblasts on the apical third region of the tooth socket form a bone matrix, and immature or woven alveolar bone begins from the apical region of the socket to the lateral wall of the socket on day 7 and then extending to the center of the socket leading to the meeting of trabecular bones. Along with the healing process of alveolar bone after the complete tooth extraction, the area of woven alveolar bone will be greater.[24] 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 areaseven had been getting greater in all groupsfrom day 7 to day 14.

Furthermore, angiogenesis is a key component in 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 normal aveolarbone 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 then 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 synthesize 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 groupsof 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 posttooth extraction conditions, 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 contrary, the number of VEGF expressionsin 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 even significantly different between that on the 7th day and that on the 14th day. This indicates that the process of angiogenesis in the treatment groups supports the process of alveolar mineralization.

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 EGF, FGF, PDGF, TGF-β1, VEGF, BMP-2, and collagen type 1.[8,9,25] Hence, in this study the VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days in the groups with the administration of the chitosan scaffold and those in the groups with the administration of chitosan-aloe vera scaffold wereincreasing and significantly different from those in the control groups.Besides, the results of this study also revealed that VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th 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 increased of VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days even 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.[26,27] HIF -1 then 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 that 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.[16,26]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.[26,28]Bonding component of the lectin protein (Aloktin) with Aloe vera polysaccharides will activate the complement system and increase coagulation to prevent loss of blood clots in bone healing.[29,30] The interactions of the protein components, such as lectin, polysaccharides, anthraquinone, and beta-sitosterol then are identified as angiogenetic factors in the healing process since they stimulate Human Umbilical Vein Endothelial Cells (HUVEC). [26, 31]Polysaccharides and flavonoids contained in Aloe vera can also increase angiogenic factors in BMSCs.[32,33]

The administration of aloe vera to tooth sockets and alveolar bone defects can increase the expression of Runx2 genes that play a role in inducing pre osteoblast differentiation into mature osteoblasts. As osteoblasts increase, the expression of OPG released by osteoblasts increases, so does ALP activity. As a result, osteoclastogenesis can be prevented through RANKL/RANK/OPG system signals. The Runx2 gene then induces osteoblasts to secrete osteopontin, osteocalcin, and type 1 collagen which influence the mineralization and bone healing process.[31,33] Therefore, it can be concluded that chitosan-Aloe vera scaffold has pore characteristics that can allow good vascularization and also accelerate alveolar bone healing process of tooth extraction in Caviacobaya through increasing VEGF expressions and the width of woven alveolar bone areasfrom day 7 to day 14.

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FORMAT PENILAIAN NASKAH DENTAL JOURNAL HASIL PENELITIAN (untuk Penyunting Ahli)

Judul Naskah: **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**

Tanggal Kirim : 22 Juni 2020   Tanggal Kembali ke Redaksi : 23 Juni 2020

HAL YANG DISUNTING	YA*	TIDAK*
1. Apakah naskah ini pernah dimuat pada media lain ? Keterangan:		v
2. Apakah judul tepat, singkat, jelas, dan menggambarkan kontribusi pengembangan keilmuan ? (maksimal 10 kata, melingkupi variabel yang diteliti) Keterangan:	v	
3. Apakah pada naskah hasil penelitian :		
a) Pendahuluan mencakup latar belakang secara jelas ? Keterangan: - Latar belakang awal tentang kehilangan gigi sebenarnya tidak relevan dengan penelitian. - Lebih tekankan latar belakang pada pentingnya soket perservation, bahan gold standard apa dan apa kekurangan bahan soket preservation yang sudah ada sehingga perlu dilakukan penelitian untuk pengembagan bahan baru - Socket preservation bukan perawatan periodontal, sebaiknya dalam penelitian jangan dikotak2 lagi	v	
b) Tujuan cukup jelas ? Keterangan:	v	
c) Metode dan rancangan penelitian sesuai dengan tujuan penelitian ? Keterangan: - Alinea pertama tentang desain penelitian (alinea 2 jadikan alinea 1) baru kemudian tahapan penelitian	v	

d) Prosedur penelitian diuraikan secara tepat dan rinci, sehingga menjamin validitas internal/ eksternal Keterangan: …………………………………………………………………………………………	v	
e) Hasil penelitian dapat menjawab *research question* ? Keterangan: …………………………………………………………………………………………	v	
f) - Pembahasan tidak mengulang hasil ? - Selaras dengan lingkup penelitian dan dibandingkan dengan hasil penelitian sejenis ? - Menerangkan makna hasil penelitian dalam menjawab permasalahan ? Keterangan: …………………………………………………………………………………………	v	
g) Acuan selaras dengan materi penelitian dan menggunakan literatur 10 tahun terakhir? Keterangan: Masih ada literatur yang lbh dari 10 tahun mohon diganti yang lebih baru karena penelitian tentang tulang sangat banyak		v
h) Kesimpulan sesuai dengan judul, permasalahan? - Hasil penelitian memberi kontribusi untuk pengembangan Ilmu kedokteran gigi ? - Melakukan sintesis berdasar hasil penelitian sejenis yang mendahului Keterangan: Hasil penelitian ini hanya bisa sampai peningkatan VEGF dan woven bone		
i) Pustaka perlu ditambahi/ dikurangi**)? Keterangan: Cukup, tahun artikel dipilih yang lebih update		
4. Apakah ada bagian yang perlu ditambahi/ diringkas**) ? Keterangan: - Latar belakang perlu diringkas lebih detil pada pentingnya mempertahankan soket, bahan soket standar yang digunakan, kerugiannya dan alasan perlu bahan baru yang diteliti ini		

REKOMENDASI untuk KETUA PENYUNTING

[.........] 1. Naskah dapat dimuat tanpa perubahan.
[.....v...] 2. Naskah dapat dimuat dengan perbaikan sesuai dengan arahan Penyunting Ahli (saran perbaikan mohon ditulis langsung pada naskah)
Keterangan:
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Alasan: ………..…………………….............................……..……………
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Penyunting Ahli,

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

ABSTRACT

Background: Microporosity and pore size of scaffold affect cellular activity, stimulate angiogenetic factors of Vascular Endothel Growth Factor (VEGF) released by endothelial cells, and also synthesize new blood vessels to regulate the migration, proliferation, and new bone formation. ***Purpose:*** This study aims to analyze the pore size of chitosan-Aloe vera scaffold and its effects on VEGF expression and woven alveolar bon
extraction of Cavia cobaya. ***Methods:*** thirty-six male Cavia cobaya, aged 3
divided into three groups, with each group consisting of 12 Cavia cobaya:
control groups (without scaffold administration), group II: positive co
chitosan scaffold administration), and group III: treatment groups (with
scaffold administration). The scaffold was applied to the sockets. Six Cavi
group were sacrificed after 7 and 14 days. The mandibular bone was cut
examination was performed to account the woven alveolar
immunohistochemical examination was conducted to analyze of VEGF ex
The largest pore size of chitosan-Aloe vera scaffold was 139.9 µm, whi
was 110.5 µm. The average pore size was 124.85 µm. It was
interconnectivity in the chitosan-Aloe vera scaffold. The use of Chitosan-
could increase VEGF expressions and the width of woven alveolar bone areas on the 7^{th} and 14^{th} days observation. Statistically, there was a significant difference between control groups and the treatment groups with chitosan- Aloe vera scaffold ($p < 0.05$). ***Conclusion:*** Chitosan-Aloe vera scaffold has pore characteristics ~~that can allow good vascularization and also accelerate alveolar bone healing process of tooth extraction in Cavia cobaya through~~ increas~~ing~~ed VEGF expressions and the width of woven alveolar bone areas.

- Describe which tooth was extracted
- SEM analysis?
- Describe statistical analysis and SPSS? Used in this study.
- In result: p value test that should be written

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

INTRODUCTION

Based on the Health Ministry's Research and Health Agency survey report in 2018, the average M-T (missing teeth) index in Indonesia was 2.5. This means that the average number of tooth lost in Indonesia was 250 teeth per 100 people indicating on average every person in Indonesia lost 3 teeth.[1] Besides, it is also known that the case of tooth loss is due to the high prevalence of periodontal disease in Indonesia, which ranks second in dental health problems in Indonesia.[2] For instance, alveolar bone resorption often occurs after tooth extraction.[3] Alveolar bone resorption then will keep staying, and even can cause more than

40% - 60% of ridge volume lose during the first 3 years post tooth extraction.[3,4] The damage of alveolar bone, unfortunately can cause failure or the instability of denture or dental implant placement.[4,5]

One of the periodontal treatments to preserve tooth sockets to regenerate alveolar bone and prevent alveolar bone resorption is by using bone graft material on bone defects.[6] 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 osteoblast cells, fibroblast cells, and type I collagen on the 7th and 14th days of observation.[8] Chitosan gel 1% 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 days 7,14, and 21.[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 healing process. Its compounds protein (alloktin), amino acids, enzymes, alkaloids, flavonoids, saponins, collagen, vitamins, calcium, potassium, and polysaccharides mannan.[10,11,12] Hence, in the previous study, the use of Aloe vera scaffold containing acemannan was increase BMSCs, VEGF, and BMP-2 proliferations, ALP activity, bone sialoprotein, mineralization, and osteopontin expressions on bone healing of tooth extraction. Aloe vera can be considered as a natural candidate for bone regeneration.[13]

Therefore, scaffold made of the combination of chitosan and Aloe vera is assumed to have a synergistic effect on tooth extraction sockets to regenerate alveolar bone and prevent alveolar bone resorption. Chitosan is osteoconduction that can support the attachment of bone-forming cells. Meanwhile, Aloe vera is osteoinduction and osteogenesis that can stimulate the differentiation of osteoprogenitor cells into osteoblast cells and also can trigger new bone formation and bone regeneration.

Microporosity structure and pore size of scaffold are known to be able to affect cellular activities, including stimulating new cell growth, cell adhesion as well as supporting cell proliferation and angiogenetic factor so that it will accelerate bone healing process. VEGF is the most dominant growth factor considered as an angiogenetic factor released by endothelial cells, which can synthesize new blood vessels to regulate the migration, proliferation, and differentiation processes of endothelial cells and the formation of new bone.[15,16] Thus, this study aims to analyze 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 on the 7th and 14th days of observation.

MATERIALS AND METHODS

Chitosan powder used in this study was chitosan powder with a deacetylation degree of > 75-85% and a molecular weight of 50,000-190,000 Da (Sigma, Product number: 448869, Lot number: MKBH7256V). Chitosan gel 1% (w/p) was made by dissolving 1 gram of chitosan powder in 100 mL of acetic acid (CH3COOH) at a concentration of 2%. After that, it was stirred using a magnetic stirrer, neutralized with NaOH solution, centrifuged at a speed of 2000 rpm for 30 minutes, and then filtered with filter paper. Aloe vera extract gel was made by maceration method. Aloe vera was cleaned, and its thorns were removed. Its gel was taken. The Aloe vera gel was blended until smooth, dried with a Freeze dry device, dissolved with 70% ethanol, and then stirred for 30 minutes with a magnetic stirrer. The maceration results were filtered with a buctner funnel coated with filter paper and accommodated with erlenmeyer. The filtered filtrate was evaporated with a vacuum rotary evaporator, and then dissolved using 3.5% Sodium carboxymethyle 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 then was put into the scaffold mold after it was put in freezer at a temperature of -80 degrees for 24 hours. Afterwards, freeze drying was carried out at a temperature of 95-103 degrees for 72 hours. The scaffold then was removed from the mold and sterilized with a UV clean bench sterilizer. Scaffold pore size examination was performed with a Scanning Electron Microscope (SEM) tool (JCM-5700, JEOL, Tokyo, Japan) with 250x and 500x magnification.

This study was an experimental research with randomized post test only control group design. Ethical Approval for this research was obtained from the Ethical committee of Airlangga University Faculty of Dentistry no 012 / HRECC.FODM / III / 2018. In this study, experimental animals used were thirty-six male Cavia cobaya , aged 3 - 3.5 months and weighed 300- 375 grams. Those Cavia cobaya animals were divided into three groups, with each group consisting of 12 Cavia cobaya: Group I: negative control groups (without scaffold administration), group II: positive control groups (with chitosan scaffold administration), and group III: treatment groups (with chitosan-Aloe vera scaffold administration). Six Cavia cobaya from each group were sacrificed after 7 and 14 days. Tooth extraction was performed on the left mandibular incisor. The tooth socket then was irrigated with sterile aquadest liquid. After that, the sterilized scaffold was applicated in the tooth socket to the apical end of the tooth, and sutured with non resorbable sutures. Those animals then were decaputated on days 7 and 14 after the treatment. Afterwards, the jaw bone in the interdental region of the mandibular incisors was cut and inserted in a fixation solution using formalin buffer 10%. Decalcification process then was carried out with EDTA for 4 weeks. Subsequently, paraffin blocks were made. Histopathological examination then was conducted with hematoxylin eosin (HE) staining to account the wid as using Image Raster 3 software. The immunohistochemical th DAB chromogen kit on the Monoclonal Anti-Cavia coba th Factor Antibody to measure the VEGF expressions in the ap

Rearange materials and methods

- Research design….
- Research sequence: scaffold manufacturing, animal model preparation and experiment, SEM analysis, HE and IHC
- Statistical analysis

Data analysis was performed using normality st. Homogeneous variation test then was conducted to find ou th Levene's test at a significance of 5%. If the data wa nd homogeneous data variations, a statistical analysis of variance analysis would be carried out and continued with a multiple comparison LSD test ($p < 0.05$). But, if the data were not normally distributed, the Kruskal-Walis non-parametric test was performed, and continued with Wilcoxon-Mann Whitney analysis to determine the different pairs of the groups.

Rearange result sequence in consistency to methods

RESULTS

SEM Test Results

The results of t oe vera scaffold with 250x and 500x magnifications showed 139.9 µm, the smallest scaffold pore size of 110.5 µm, and .85 µm. It was found a good pore interconnection or open an-Aloe vera scaffold (Figure 1).

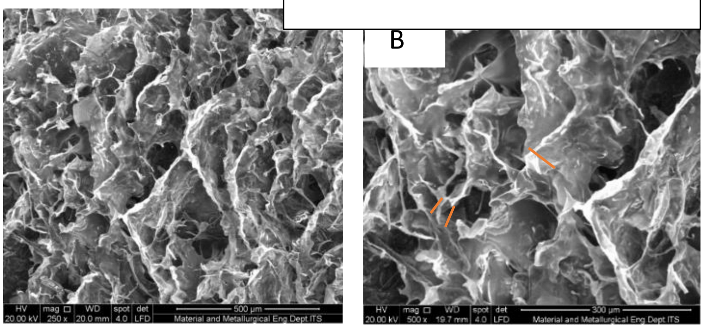


Figure 1. SEM test results on the pore size of the chitosan-Aloe vera scaffold with the magnifications of 250x (a) and 500x (b)

- Describe IHC findings in each group according to figure shown.

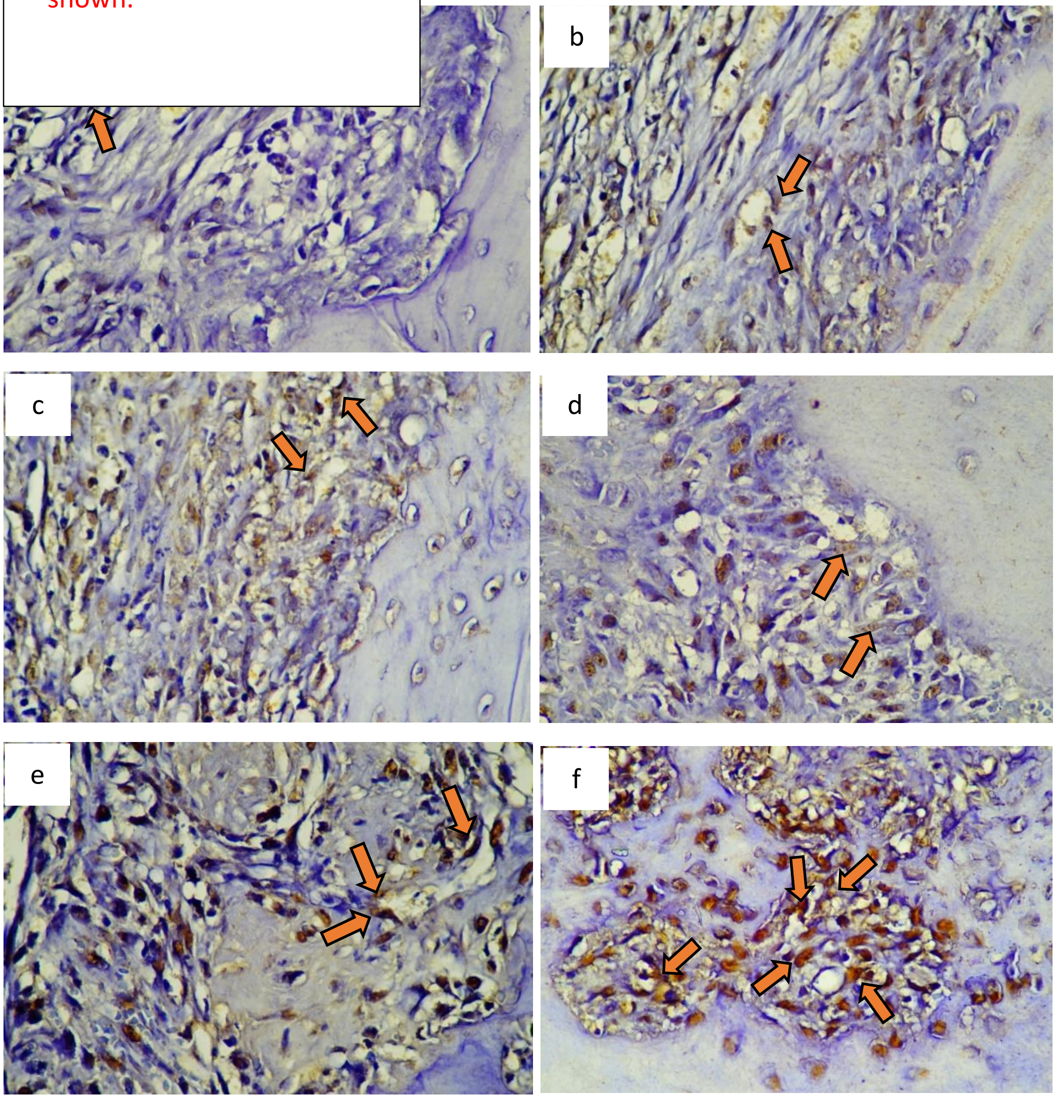
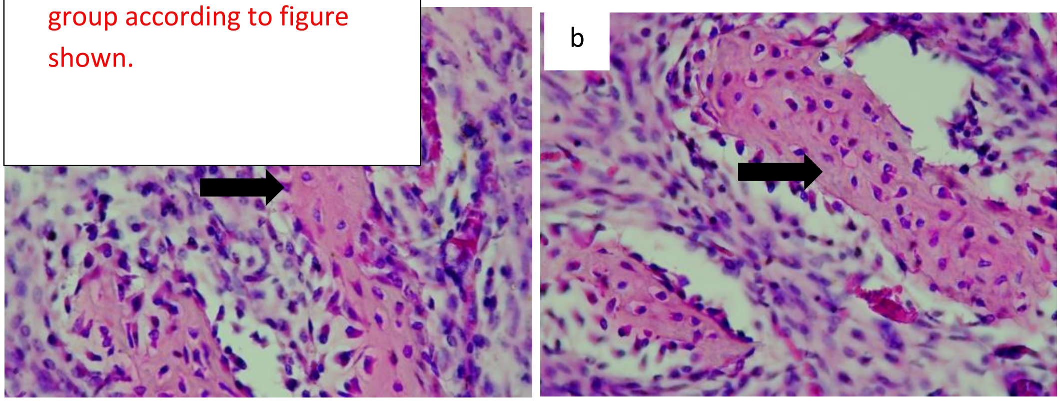


Figure 2. VEGF expressions on endothelial cells showing brown color marked with arrows. (a) The control group on day 7, (b) The control group on day 14, (c) The treatment group with chitosan scaffold on day 7, (d) The treatment group with chitosan scaffold on day 14, (e) The treatment group with chitosan-Aloe vera scaffold on day 7, (f) The treatment group with chitosan-Aloe vera scaffold on day 14, with 400x magnification

- Describe HE findings in each group according to figure shown.



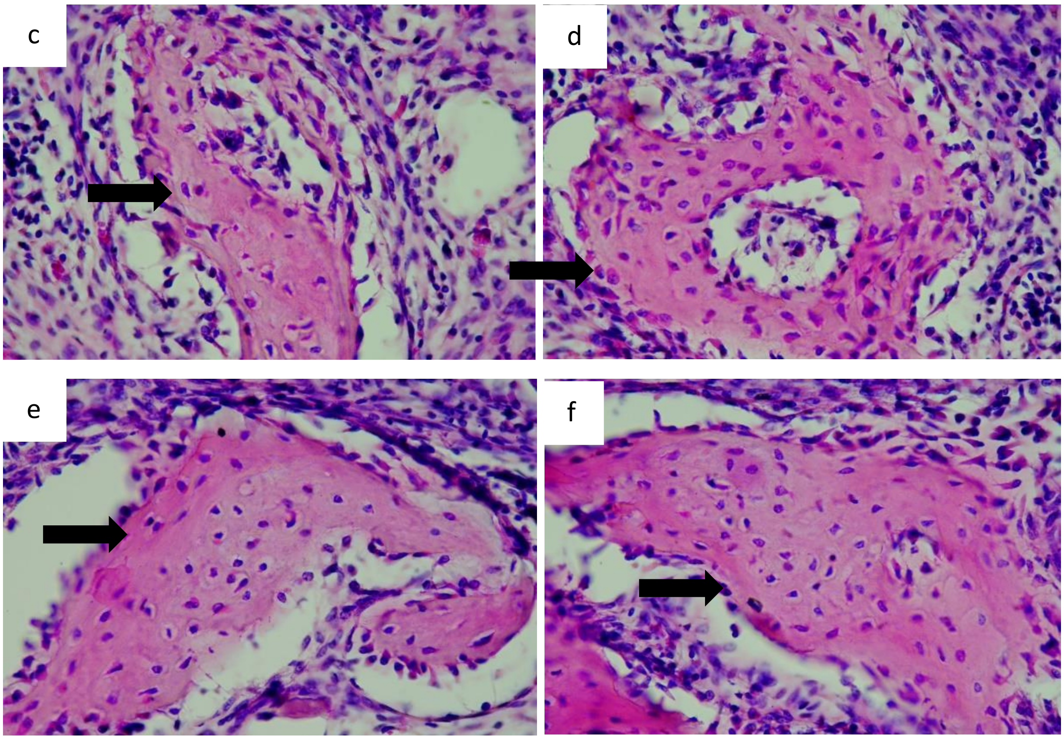


Figure 3. The woven alveolar bone areas. (A) The control group on day 7, (B) The control group on day 14, (C) The treatment group with chitosan scaffold on day 7, (D) The treatment group with chitosan scaffold on day 14, (E) The treatment group with chitosan-Aloe vera scaffold on day 7, (F) The treatment group with chitosan-Aloe vera scaffold on day 14, with 100x magnification

Table 1. The mean and standard deviation (SD) of VEGF expressions in all groups

Groups	N	VEGF Expressions (cells/LP)				P
		$\bar{x}$	SD	Min	Max	
Control on day 7	6	6.50[a]	1.64	4.0	8.0	0.000*
Control on day 14	6	8.33[a]	1.75	6.0	11.0	
Chitosan on day 7	6	8.00[a]	1.79	5.0	10.0	
Chitosan on day 14	6	11.60[b]	1.72	8.3	13.0	
Chitosan+*A.vera* on day 7	6	11.50[b]	1.39	10.0	14.0	
Chitosan+*A.vera* on day 14	6	15.28[c]	1.78	13.7	18.0	

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

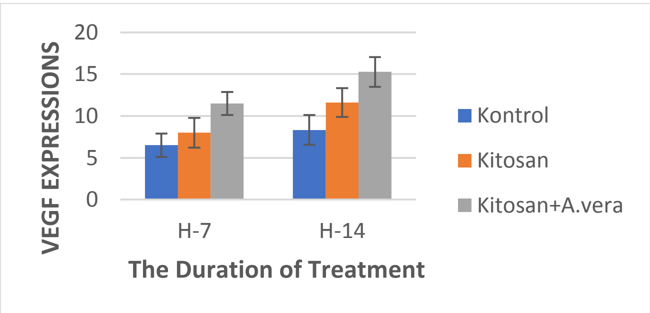


Figure 4. The Diagram of VEGF expressions in the control groups, the treatment groups with chitosan scaffold, and the treatment groups with chitosan-Aloe vera scaffold on days 7 and 14

Table 2. The mean and standard deviation of Woven Bone Areas in all groups

Groups	n	Woven Bone Areas (μm^2)					P
		$\bar{x}$	SD	Median	Min	Maks	
Control on day 7	6	10.50	1.23	11.0 [a]	9	12	0.000*
Control on day 14	6	17.83	2.99	17.5 [c]	13	21	
Chitosan on day 7	6	12.67	1.63	12.5 [b]	11	15	
Chitosan on day 14	6	27.17	5.98	26.0 [d]	21	38	
Chitosan+*A.vera* on day 7	6	17.83	1.47	18.0 [c]	15	19	
Chitosan+*A.vera* on day 14	6	37.67	6.65	35.5 [e]	32	49	

Note: * significant at $\alpha = 0.05$ (Kruskal-Wallis test)
[abcde] Different superscripts show differences between groups (Mann-Whitney test)

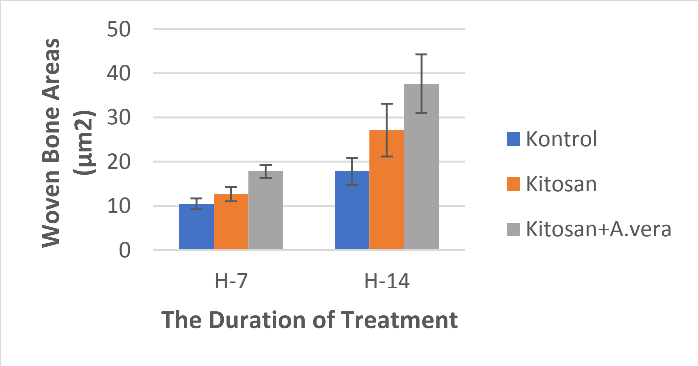


Figure 5. The Diagram of woven alveolar bone areas in the control groups, the treatment groups with chitosan scaffold, and the treatment groups with chitosan-Aloe vera scaffold on days 7 and 14

The results of the analysis showed that the use of chitosan-Aloe vera scaffold could significantly increase the VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days compared with the control group and the group with application of chitosan scaffold (table 1 and 2).

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 bone healing process. The single use of chitosan as scaffold has unadequate pore size, poor porosity, and close interconnectivity to facilitate the transportation of nutrients, growth factors, and blood vessels.[7.17,18]

Unlike the scaffold made of the single use of chitosan, scaffold made of the combination of chitosan and Aloe vera, based on the SEM test results, has a mean pore size of 124.85 μm. The chitosan-aloe vera scaffold has a good pore interconnectivity or open pore interconectivity. The recommended minimum pore size for scaffold is 100 μm, which enables the scaffold not only to provide a good micro or nich environment 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 vascularization 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 synthesize new blood vessels to regulate the migration, proliferation, and new bone formation. [19,20] In our study, the use of chitosan-Aloe vera scaffold could increase VEGF expressions as well as the width of woven alveolar bone areas on the 7th and 14th day compared to the use of chitosan scaffold.

Moreover, alveolar bone healing process of tooth extraction actually begins with hemostasis 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 PDGF and TGF-β. These molecules can activate and attract PMNs, macrophages, and endothelial cells to the socket. Macrophage cells 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.[21,22] In the final inflammatory phase, macrophage cells begins to stimulate increasing of induced growth factors as PDGF, FGF, VEGF, TGF-β, and TGF-α.[22,23] VEGF is the most dominant angiogenetic factors released by endothelial cells to synthesize 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 even was not 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 maximized yet.

Differentiated osteoblasts on the apical third region of the tooth socket form a bone matrix, and immature or woven alveolar bone begins from the apical region of the socket to the lateral wall of the socket on day 7 and then extending to the center of the socket leading to the meeting of trabecular bones. Along with the healing process of alveolar bone after the complete tooth extraction, the area of woven alveolar bone will be greater.[24] 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 even had been getting greater in all groups from day 7 to day 14.

Furthermore, angiogenesis is a key component in 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 normal aveolar 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 then 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 synthesize 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, 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 contrary, 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 even significantly different between that on the 7th day and that on the 14th day. This indicates that the process of angiogenesis in the treatment groups supports the process of alveolar mineralization.

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 EGF, FGF, PDGF, TGF-β1, VEGF, BMP-2, and collagen type 1.[8,9,25] Hence, in this study the VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th 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.Besides, the results of this study also revealed that VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th 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 increased of VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days even 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.[26,27] HIF -1 then 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 that 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.[16,26] 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.[26,28] Bonding component of the lectin protein (Aloktin) with Aloe vera polysaccharides will activate the complement system and increase coagulation to prevent loss of blood clots in bone healing.[29,30] The interactions of the protein components, such as lectin, polysaccharides, anthraquinone, and beta-sitosterol then are identified as angiogenetic factors in the healing process since they stimulate Human Umbilical Vein Endothelial Cells (HUVEC). [26, 31] Polysaccharides and flavonoids contained in Aloe vera can also increase angiogenic factors in BMSCs.[32,33]

The administration of aloe vera to tooth sockets and alveolar bone defects can increase the expression of Runx2 genes that play a role in inducing pre osteoblast differentiation into mature osteoblasts. As osteoblasts increase, the expression of OPG released by osteoblasts increases, so does ALP activity. As a result, osteoclastogenesis can be prevented through RANKL/RANK/OPG system signals. The Runx2 gene then induces osteoblasts to secrete osteopontin, osteocalcin, and type 1 collagen which influence the mineralization and bone healing process.[31,33] Therefore, it can be concluded that chitosan-Aloe vera scaffold has pore characteristics that can ~~allow good vascularization and also accelerate alveolar bone healing process of tooth extraction in Cavia cobaya through increasing~~ increased VEGF expressions and the width of woven alveolar bone areas ~~from day 7 to day 14~~.

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DAFTAR TILIK *MANAGING EDITOR*

Judul Naskah: **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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FORMAT	
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Pendahuluan	
▪ Latar belakang empirik/teoritik	Ada/Tidak
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Bahan dan Metode	
▪ Rancangan (jenis, masa (waktu), tempat penelitian)	Ada/ Tidak
▪ Teknik pengambilan sampel	Ada/ Tidak
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HAL YANG DISUNTING	KETERANGAN*)
▪ Analisa data	Ada/ Tidak
Hasil	
▪ Paparan data	Ada/ Tidak
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▪ Pembahasan tidak mengulang hasil	Ya/ Tidak
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Catatan:

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REKOMENDASI *MANAGING EDITOR* (PILIH SALAH SATU)

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Surabaya,

Research Report

The Pore size of Chitosan-Aloevera Scaffold and its effect on VEGF expressions and woven alveolar bone healing of tooth extraction of Caviacobaya

ABSTRACT

Background: Microporosityand pore size of scaffold affect cellular activity, stimulate angiogenetic factors of Vascular Endothel Growth Factor (VEGF) released by endothelial cells, and also synthesize new blood vessels to regulate the migration, proliferation, and new bone formation.***Purpose:*** This study aims to analyze the pore size of chitosan-Aloe vera scaffold and its effects on VEGF expression and woven alveolar bone healing of tooth extraction of Caviacobaya. ***Methods:*** thirty-six male Cavia cobaya, aged 3 - 3.5 months were divided into three groups, with each group consisting of 12 Cavia cobaya: Group I: negative control groups (without scaffold administration), group II: positive control groups (with chitosan scaffold administration), and group III: treatment groups (with chitosan-Aloe vera scaffold administration). The scaffold was applied to the sockets. Six Cavia cobaya from each group were sacrificed after 7 and 14 days.The mandibular bone was cut. Histopathological examination was performed to account the woven alveolar bone areasand immunohistochemical examination was conducted to analyze of VEGF expressions. ***Results:***The largest pore size of chitosan-Aloe vera scaffold was 139.9 µm, while the smallestone was 110.5 µm. The average pore size was 124.85 µm. It was found open pore interconnectivity in the chitosan-Aloe vera scaffold. The use of Chitosan-Aloe verascaffold could increase VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days observation. Statistically,there was a significant difference between control groups and the treatment groups with chitosan- Aloe verascaffold ($p < 0.05$). ***Conclusion:*** Chitosan-Aloe vera scaffold has pore characteristics that can allow good vascularization and also accelerate alveolar bone healing processof tooth extraction in Caviacobaya through increasing VEGF expressions and the width of woven alveolar bone areas.

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

Commented [ks1]: ... vascular endothelial growth factor (VEGF)

Commented [ks2]: Analisa statistik yang digunakan harap ditulis dengan nilai signifikansinya

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INTRODUCTION

~~Based on the Health Ministry's Research and Health Agency survey report in 2018, the average M-T (missing teeth) index in Indonesia was 2.5. This means that the average number of tooth lost in Indonesia was 250 teeth per 100 people indicating on average every person in Indonesia lost 3 teeth.[1] Besides, it is also known that the case of tooth loss is due to the high prevalence of periodontal disease in Indonesia, which ranks second in dental health problemsin Indonesia.~~[2]For instance, alveolar bone resorption often occurs after tooth extraction.[3] Alveolar bone resorption then will keep staying, and even can cause more than

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40% - 60% of ridge volume lose during the first 3 years post tooth extraction.[3,4]The damage of alveolar bone, unfortunatelycan cause failure or the instability of denture or dental implantplacement.[4,5]

One of the periodontal treatments to preserve tooth sockets to regenerate alveolar bone and prevent alveolar bone resorption is by using bone graft material on bone defects.[6]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 norvegicuscan increase the number of osteoblast cells, fibroblast cells, and type I collagen on the 7th and 14th days of observation.[8] Chitosan gel 1% is also known to be able to increase Bone Morphogenetic Protein-2 (BMP-2) expressionsof Rattus norvegicus during bone formation after tooth extraction on days 7,14, and 21.[9]

Aloe verais 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 healing process. Its compounds protein (alloktin), amino acids, enzymes, alkaloids, flavonoids, saponins, collagen, vitamins, calcium, potassium, and polysaccharides mannan.[10,11,12]Hence, in the previous study, the use of Aloe verascaffold containing acemannanwas increase BMSCs, VEGF, and BMP-2proliferations, ALP activity, bone sialoprotein, mineralization, and osteopontin expressions on bone healing of tooth extraction. Aloe veracan be considered as a natural candidate for bone regeneration.[13]

Therefore, scaffold made of the combination of chitosan and Aloe verais assumed to have a synergistic effect on tooth extraction sockets to regenerate alveolar bone and prevent alveolar bone resorption. Chitosan is osteoconductionthat can support the attachment of bone-forming cells.Meanwhile, Aloe vera is osteoinduction and osteogenesisthat can stimulate the differentiation of osteoprogenitor cells into osteoblast cells and also can trigger new bone formation and bone regeneration.

Microporositystructure and pore size of scaffold are known to be able to affect cellular activities, including stimulating new cell growth, cell adhesion as well as supporting cell proliferation and angiogenetic factor so that it will accelerate bone healing process. VEGF is the most dominant growth factor considered as an angiogenetic factor released by endothelial cells,which can synthesize new blood vessels to regulate the migration, proliferation, and differentiation processes of endothelial cells and the formationof new bone.[15,16] Thus, this study aims to analyze the pore size of chitosan-Aloe vera scaffold and its

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Commented [ks8]: Ditulis lengkap terlebih dahulu seblum singkatan (kalau istilah ini muncul lagi di bagian lain dari manuscript). Kalau hanya muncul sekali, tdk perlu diberi singkatan effects on VEGF expression and woven alveolar bone healing of tooth extraction of Caviacobayaon the 7th and 14th days of observation.

MATERIALS AND METHODS

Chitosan powder used in this studywas chitosan powder with a deacetylation degree of > 75-85%and a molecular weight of 50,000-190,000 Da (Sigma, Product number: 448869, Lot number: MKBH7256V). Chitosan gel 1% (w/p) was made by dissolving 1 gram of chitosan powder in 100 mL of acetic acid (CH3COOH) at a concentration of 2%. After that, it was stirred using a magnetic stirrer, neutralized with NaOH solution, centrifuged at a speed of 2000 rpm for 30 minutes,and then filtered with filter paper. Aloe vera extract gel was made by maceration method.Aloe verawas cleaned, and its thorns were removed. Its gel was taken. The Aloe vera gel was blended until smooth, dried with a Freeze dry device, dissolved with 70% ethanol, and then stirred for 30 minutes with a magnetic stirrer. The maceration results were filtered with a buctner funnel coated with filter paper and accommodated with erlenmeyer. The filtered filtrate was evaporated with a vacuum rotary evaporator, and then dissolved using 3.5% Sodium carboxymethyle 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 then was put into the scaffold mold after it was put in freezer at a temperature of -80 degrees for 24 hours.Afterwards, freeze drying was carried out at a temperature of 95-103 degrees for 72 hours. The scaffold then was removed from the mold and sterilized with a UV clean bench sterilizer. Scaffold pore size examination was performed with a Scanning Electron Microscope (SEM) tool (JCM-5700, JEOL, Tokyo, Japan) with 250x and 500x magnification.

This study was an experimental research with randomized post test only control group design. Ethical Approval for this research was obtained from the Ethical committee of Airlangga University Faculty of Dentistry no 012 / HRECC.FODM / III / 2018. In this study, experimental animals used were thirty-six male Caviacobaya,aged 3 - 3.5 monthsand weighed 300- 375 grams. Those Caviacobayaanimals were divided into three groups, with each group consisting of 12 Caviacobaya: Group I: negative control groups (without scaffold administration), group II: positive control groups (with chitosan scaffold administration), and group III: treatment groups (with chitosan-Aloe vera scaffold administration). SixCaviacobaya from each group were sacrificed after 7 and 14 days.Tooth extraction was performed on the left mandibular incisor. The tooth socket then was irrigated with sterile

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Commented [ks13]: ... scanning electron microscope (SEM) aquadest liquid. After that, the sterilized scaffold was applicated in the tooth socket to the apical end of the tooth, and sutured with non resorbable sutures. Those animals then were decaputated on days 7 and 14 after the treatment. Afterwards, the jaw bone in the interdental region of the mandibular incisors was cut and inserted in a fixation solution using formalin buffer10%. Decalcification process then was carried outwith EDTA for 4 weeks. Subsequently, paraffin blocks were made. Histopathological examination then was conductedwith hematoxylin eosin (HE) staining to account the width of woven alveolar bone areas using Image Raster 3 software.The immunohistochemical examination was performed with DAB chromogen kit on the Monoclonal Anti-Caviacobaya Vascular Endothelial Growth Factor Antibody to measure the VEGF expressions in the apical third of teeth.

Data analysis was performed using normality test with Shapiro Wilk test. Homogeneous variation test then was conducted to find out data variation inthe groups with Levene's test at a significance of 5%. If the data was normally distributed and had homogeneous data variations, a statistical analysis of variance analysis would be carried out and continued with a multiple comparison LSD test ($p < 0.05$). But, if the data were not normally distributed, the Kruskal-Walis non-parametric test was performed, and continued with Wilcoxon-Mann Whitney analysis to determine the different pairs of the groups.

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RESULTS

~~**SEM Test Results**~~

The results of the SEM test on the chitosan-Aloe vera scaffold with 250x and 500x magnifications showed the largest scaffold pore size of 139.9 µm, the smallest scaffold pore size of 110.5 µm, and the average pore size of 124.85 µm. It was found a good pore interconnection or open pore interconnectivity of chitosan-Aloe vera scaffold(Figure 1).

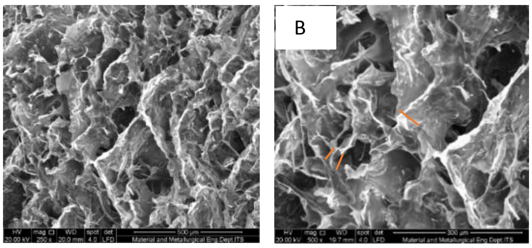


Figure 1. SEM test results on the pore size of the chitosan-Aloe vera scaffold with the magnifications of 250x (a) and 500x (b)

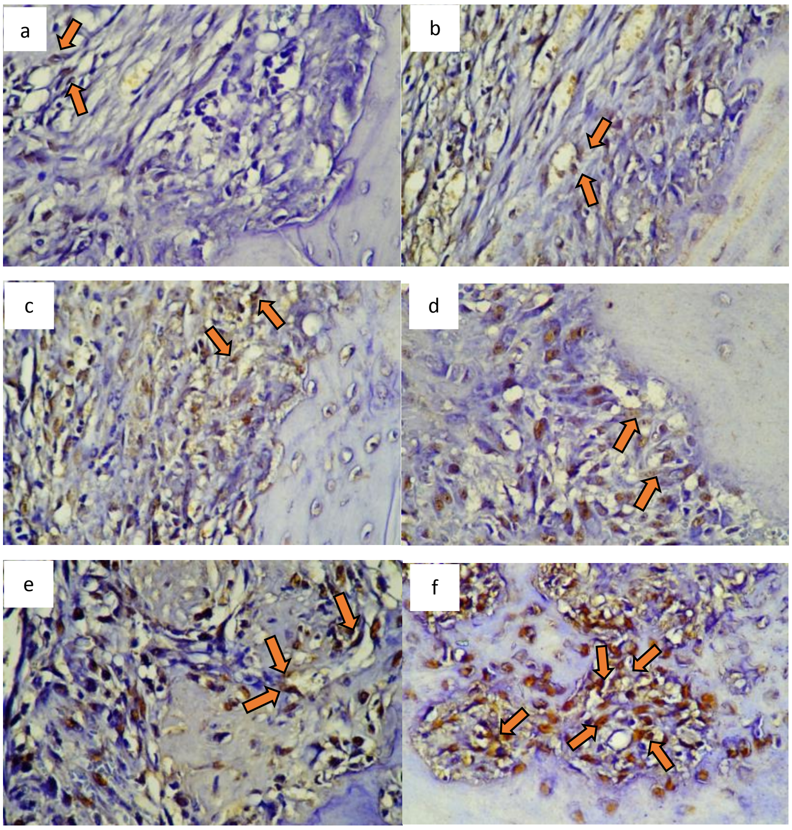
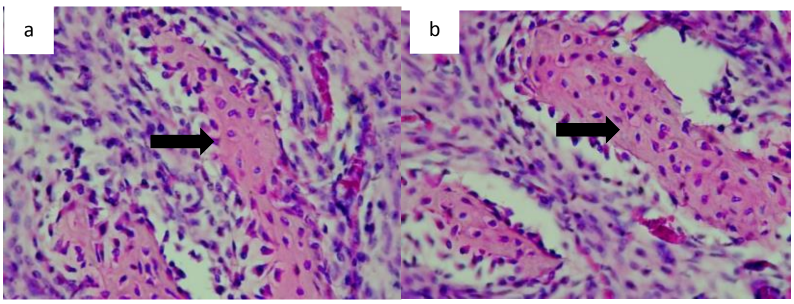


Figure 2. VEGF expressions on endothelial cells showing brown color marked with arrows. (a) The control group on day 7, (b) The control group on day 14, (c) The treatment group with chitosan scaffold on day 7, (d) The treatment group with chitosan scaffold on day 14, (e) The treatment group with chitosan-Aloe vera scaffold on day 7, (f) The treatment group with chitosan-Aloe vera scaffold on day 14, with 400x magnification



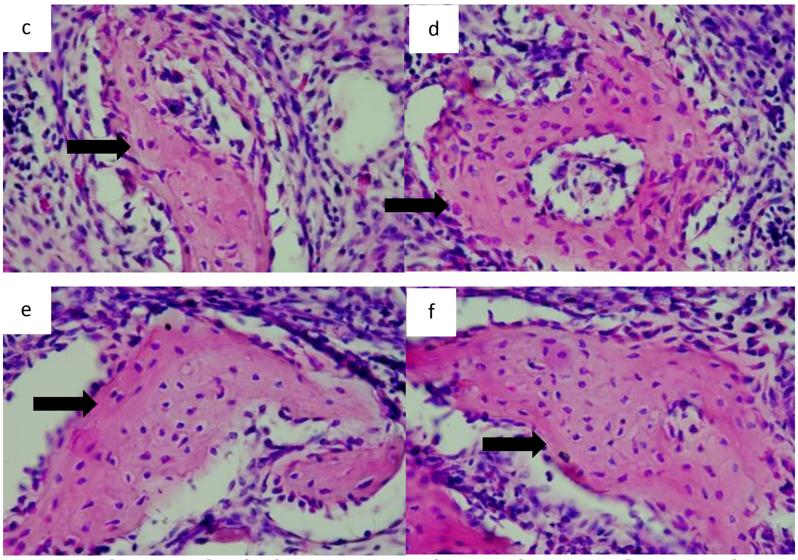


Figure 3. Thewoven alveolar bone areas. (A) The control group on day 7, (B) The control group on day 14, (C) The treatment group with chitosan scaffold on day 7, (D) The treatment group with chitosan scaffold on day 14, (E) The treatment group with chitosan-Aloe vera scaffold on day 7, (F) The treatment group with chitosan-Aloe vera scaffold on day 14, with 100x magnification

Table1. The mean and standard deviation (SD)of VEGF expressions in all groups

Groups	N	VEGF Expressions (cells/LP)				P
		$\bar{x}$	SD	Min	Max	
Control on day 7	6	6.50[a]	1.64	4.0	8.0	0.000*
Control on day 14	6	8.33[a]	1.75	6.0	11.0	
Chitosan on day 7	6	8.00[a]	1.79	5.0	10.0	
Chitosan on day 14	6	11.60[b]	1.72	8.3	13.0	
Chitosan+*A.vera* on day 7	6	11.50[b]	1.39	10.0	14.0	
Chitosan+*A.vera* on day 14	6	15.28[c]	1.78	13.7	18.0	

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

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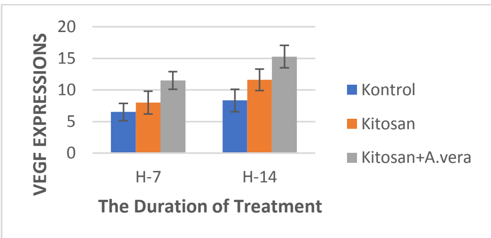

Figure 4. The Diagram ofVEGF expressions in the control groups, the treatment groups with chitosan scaffold, and the treatment groups with chitosan-Aloe vera scaffold on days 7 and 14

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Table 2. The mean andstandard deviation of Woven Bone Areas in all groups

Groups	n	Woven Bone Areas (μm^2)					P
		$\bar{x}$	SD	Median	Min	Maks	
Control on day 7	6	10.50	1.23	11.0 [a]	9	12	0.000*
Control on day 14	6	17.83	2.99	17.5 [c]	13	21	
Chitosan on day 7	6	12.67	1.63	12.5 [b]	11	15	
Chitosan on day 14	6	27.17	5.98	26.0 [d]	21	38	
Chitosan+*A.vera* on day 7	6	17.83	1.47	18.0 [c]	15	19	
Chitosan+*A.vera* on day 14	6	37.67	6.65	35.5 [e]	32	49	

Note: * significant at α = 0.05 (Kruskal-Wallis test)
[abcde] Different superscripts show differences between groups (Mann-Whitney test)

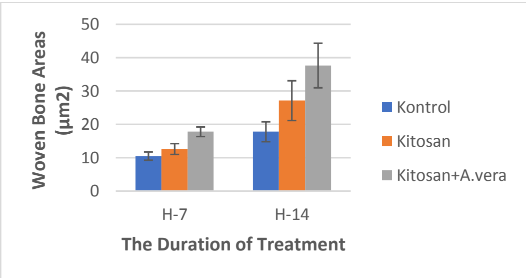

Figure 5. The Diagramof woven alveolarbone areas in the control groups, the treatment groups with chitosan scaffold, and the treatment groups with chitosan-Aloe vera scaffold on days 7 and 14

The results of the analysis showed that the use of chitosan-Aloe vera scaffold could significantly increase theVEGF expressions and the width of woven alveolarbone areas on the 7th and 14th days compared with the control group and the group with application of chitosan scaffold (table 1 and 2).

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 bone healing process. The single use of chitosan as scaffold has unadequate pore size, poor porosity, and close interconnectivity to facilitate the transportation of nutrients, growth factors, and blood vessels.[7,17,18]

Unlike the scaffold made of the single use of chitosan, scaffold made of the combination of chitosan and Aloe vera,based on the SEM test results,has a mean pore size of 124.85 µm. The chitosan-aloe vera scaffold has a good pore interconnectivity or open pore interconectivity. The recommended minimum pore size for scaffold is 100 µm,which enables

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Moreover, alveolar bone healing process of toothextraction actually begins with hemostasis 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 PDGF and TGF-β. These molecules can activate and attract PMNs, macrophages, and endothelial cells to thesocket. Macrophage cells 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.[21,22]In the final inflammatory phase, macrophage cells begins to stimulate increasing of induced growth factors as PDGF, FGF, VEGF, TGF-β, and TGF-α.[22,23] VEGF is the most dominant angiogenetic factors released by endothelial cells to synthesize 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 even was not 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 maximized yet.

Differentiated osteoblasts on the apical third region of the tooth socket form a bone matrix, and immature or woven alveolar bone begins from the apical region of the socket to the lateral wall of the socket on day 7 and then extending to the center of the socket leading to the meeting of trabecular bones. Along with the healing process of alveolar bone after the complete tooth extraction, the area of woven alveolar bone will be greater.[24] 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 areaseven had been getting greater in all groupsfrom day 7 to day 14.

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Furthermore, angiogenesis is a key component in 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 normal aveolarbone 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 then 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 synthesize 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 groupsof 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 posttooth extraction conditions, 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 contrary, the number of VEGF expressionsin 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 even significantly different between that on the 7th day and that on the 14th day. This indicates that the process of angiogenesis in the treatment groups supports the process of alveolar mineralization.

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... hypoxia-inducible factor 1 (HIF-1).

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 EGF, FGF, PDGF, TGF-β1, VEGF, BMP-2, and collagen type 1.[8,9,25] Hence, in this study the VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days in the groups with the administration of the chitosan scaffold and those in the groups with the administration of chitosan-aloe vera scaffold wereincreasing and significantly different from those in the control groups.Besides, the results of this study also revealed that VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th 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 increased of VEGF expressions and the width of woven alveolar bone areas on the 7th and 14th days even 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.[26,27] HIF -1 then 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 that 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.[16,26]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.[26,28]Bonding component of the lectin protein (Aloktin) with Aloe vera polysaccharides will activate the complement system and increase coagulation to prevent loss of blood clots in bone healing.[29,30] The interactions of the protein components, such as lectin, polysaccharides, anthraquinone, and beta-sitosterol then are identified as angiogenetic factors in the healing process since they stimulate Human Umbilical Vein Endothelial Cells (HUVEC). [26, 31]Polysaccharides and flavonoids contained in Aloe vera can also increase angiogenic factors in BMSCs.[32,33]

The administration of aloe vera to tooth sockets and alveolar bone defects can increase the expression of Runx2 genes that play a role in inducing pre osteoblast differentiation into mature osteoblasts. As osteoblasts increase, the expression of OPG released by osteoblasts increases, so does ALP activity. As a result, osteoclastogenesis can be prevented through RANKL/RANK/OPG system signals. The Runx2 gene then induces

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Commented [ks25]: .. human umbilical vein endothelial cells (HUVEC).

osteoblasts to secrete osteopontin, osteocalcin, and type 1 collagen which influence the mineralization and bone healing process.[31,33] Therefore, it can be concluded that chitosan-Aloe vera scaffold has pore characteristics that can allow good vascularization and also accelerate alveolar bone healing process of tooth extraction in Caviacobaya through increasing VEGF expressions and the width of woven alveolar bone areasfrom day 7 to day 14.

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