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To: Dr Saskianti <tania-s@fkg.unair.ac.id>

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Journal Name: Clinical, Cosmetic and Investigational Dentistry
Title: Study of alveolar bone remodeling using deciduous tooth stem cells and hydroxyapatite by vascular endothelial growth factor enhancement and inhibition of matrix metalloproteinase-8 expression in vivo
ID: 354153
Author: Dr Saskianti

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Submission to Clinical, Cosmetic and Investigational Dentistry [ID 354153]

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Ms Sandi McIver <sandi@dovepress.com> Mon, Mar 14, 2022 at 2:00 AM
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Dear All Authors,

I hope everyone is healthy.
Herewith i informed you that our paper has been accepted.
I very much appreciate your support, suggestion, and guidance during the manuscript writing.
Looking forward to collaborate with all of you again in the near future.

Sincerely,

Tania
[Quoted text hidden]
--
Tania Saskianti, DDS., Ph.D., Sp.KGA(K)

Lecturer - Department of Pediatric Dentistry
Head of Research Centre & Research Groups

Faculty of Dental Medicine - Universitas Airlangga
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河本健 <tkawamo@gmail.com> Tue, Mar 15, 2022 at 3:14 PM
To: tania saskianti <tania-s@fkg.unair.ac.id>

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2022年3月13日(日) 7:47 tania saskianti <tania-s@fkg.unair.ac.id>:
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Clinical, Cosmetic and Investigational Dentistry - Your publication schedule [ID 354153]

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Mel Phimester <melanie@dovepress.com> Wed, Mar 16, 2022 at 5:53 AM
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To: tania-s@fkg.unair.ac.id

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1 message

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I would like to take this opportunity to personally thank you for your contribution to Clinical, Cosmetic and Investigational Dentistry. It was a pleasure working with you and I hope we can do so again in the near future.

Yours sincerely

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ORIGINAL RESEARCH

Tania Saskianti et al

Study of alveolar bone remodeling using deciduous tooth stem cells and hydroxyapatite~~Hydroxyapatite~~ by VEGF enhancement and inhibition of MMP-8~~MMP8~~ expression in vivo

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Alexander Patera Nugraha[2]

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Abstract

Background: Periodontitis progression is characterized by alveolar bone loss, and its prevention is a major clinical problem in periodontal disease management. MatrixThe matrix metalloproteinase-8 (MMP-8) has been shown to adequately monitor the treatment of chronic periodontitis patients as gingival crevicular fluid MMP-8sMMPs-8 were positively associated with the severity of periodontal disease. Moreover, modulating the vascular endothelial growth factor (VEGF) levels in bones could be a good way to improve bone regeneration and cure periodontitis as VEGF promotes endothelial cell proliferation, proteolytic enzyme release, chemotaxis, and migration;, all of which are required for angiogenesis to occur.

Purpose: The aim of this study was to determine the effect of hydroxyapatite (HA) incorporated with stem cellscell from exfoliated deciduous teeth (SHED) in Wistarwistar rats' initial alveolar bone remodeling basedremodelling on the findingsbasis of MMP-8 and VEGF expressionsexpression.

Methods: A hydroxyapatiteHydroxyapatite scaffold (HAS) in conjunction with SHED was transplanted into animal models withof alveolar mandibular defects. A total of 10 Wistar rats (*Rattus novergicus*) were divided into two groups:(i) HAS and; (ii) HAS + SHED.

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Immunohistochemistry staining was performed after 7 days ~~in order~~ to facilitate the examination of MMP-8 and VEGF expressions~~expression~~.

Results: The independent t-test ~~showed that downregulated of MMP-8 and upregulated VEGF expression were~~ found significant downregulation of MMP-8 and upregulation VEGF expressions~~significantly~~ in groups transplanted with HAS in conjunction with SHED compared with the~~to~~ HAS group~~only~~ (p < ~~<~~0.05).

Conclusion: The combination~~Combination~~ of SHED with HAS on alveolar bone defects~~defect~~ may contribute to initial alveolar bone remodeling as evident~~remodelling~~ through the assessments of MMP-8 and VEGF expressions~~expression~~.

Keywords: angiogenesis, medicine, osteogenesis, scaffold, tissue engineering

Introduction

Periodontal disease is an infectious and inflammatory condition that damages the teeth's supporting structures through~~by~~ bone resorption and periodontal tissue loss caused by acute (sometimes violent) or chronic inflammation.[1] Periodontal disease may result in edentulism and has been linked to severe systemic disorders, including ~~as~~ atherosclerosis, cardiovascular disease, diabetes, and rheumatoid arthritis.[2–5] This may have a direct impact on afflicted individuals' general health, social life, and nutritional status, endangering their entire quality of life.[6–9] The global prevalence of periodontal disease is believed to be around 11%, which is the sixth most common human disease with a significant public health burden worldwide.[10] As a response, it is critical to provide a timely and effective therapy for periodontal disease.

The ultimate goal of periodontal therapy is to slow ~~down~~ the progression of periodontitis and enhance periodontal tissue regeneration.[11] Scaling and root planing~~planning~~, as well as periodontal surgery for periodontal tissue rebuilding, are the major treatments for periodontal tissue inflammation.[12] However, the clinical outcomes in patients with periodontal disease are not completely satisfactory~~satisfying~~ because the ~~regeneration of~~ destroyed tissue is not regenerated~~achieved~~.[13] The desired therapeutic outcome is a proper regeneration of alveolar bone, root cementum, and periodontal ligaments~~ligament~~ in the previously damaged

periodontium.[14] As a result, various therapeutic options have been proposedoffered, including stem cell–based tissue engineering and regenerative therapy.[15–17]

Among mesenchymal stem cellsMesenchymal Stem Cells (MSCs) from dental tissue, human exfoliated deciduous tooth cells (SHEDs) are prominent.[18] Dental stem cells were isolated initially isolated from the dental pulp of permanent teeth (DPSC) and then from the dental pulp of deciduous teeth (SHED).[19] Miura et al., were the first to successfully employ SHED in vivo in conjunction with a scaffold for bone tissue building applications. Other research showed that SHED and hDPSC transplantation in the calvaria of immunodeficient mice resulted in nearly the same quantity of new bone formation as human bone marrow MSCmesenchymal stem cell (hBMSC) transplantation.[20] As they originateOriginated from a more immature subpopulation than permanent teeth, SHED have a higher proliferation rate and differentiation potential since they can differentiate into neural cells, adipocytes, osteoblasts, and odontoblasts.[19] In addition, SHED areis capable of spontaneously producing large volumes of bone in vivo.[19,21] Because of the ease of availability, SHED areis excellent source of stem cells.

In addition to the sourceInstead of only the stem cell, source, certain other aspects are critical for successful tissue engineering, success such as the biomaterial to be selectedemployed as a scaffold and the correct linkage between them.[22] To regenerate the bone tissue defect, it is necessary that the selectedchosen biomaterial must allowallows cells to migrate, proliferate, and differentiate into bone cells, but it is also necessary that local angiogenesis is also requiredoccurs to provide the necessary nutrients and environmentalenvironment factors for the correct development of the bone tissue development.[23] Hydroxyapatite (HA) is a frequently used frequent biomaterial for constructingused as a scaffold. When utilized as a bone graft, HAhidroxyapatite, a key mineral component of human hard tissue that is widely used clinically to repair alveolar bone defects, is aone of the bioactive materialmaterials that also exhibits osseointegration, osteoconduction, and osteogenesis characteristics.[19,24] However, little researchnot much literature exists ontoexamine the initial alveolar bone-remodeling remodelling ability of HAhydroxyapatite as a scaffold materialmaterials used as therapy along with the use of SHED as an osteoinductive substance in alveolar bone defectsdefect.

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Commented [PI2]: Did you mean "human DPSC?" Spell out the "h."

Matrix metalloproteinase-8 (MMP-8) and vascular endothelial growth factor (VEGF) are involved in regenerative therapy with transplanted SHED in alveolar bone defectsdefect. In this study, SHED was combined with a hydroxyapatite scaffold (HAS) and transplanted onto rat models withmodel of alveolar bone defectsdefect to demonstrate the potential effects of thesethose incorporated materials on initial bone remodelingremodelling by evaluating MMPmatrix metalloproteinase-8 (MMP-8) and vascular endothelial growth factor (VEGF) expressionsexpression. Because of its high level of expression from neutrophils, MMP-8 plays a rolehas functions in initiatingbeginning collagen degradation in the extracellular matrix during embryogenesis, bone healing, and bone regeneration, as well as reflecting the inflammatory response in the first wound repair stage.[25–29] Moreover, angiogenesis is controlled by a number of growth factors, most notably VEGF, which is produced by inflammatory and stromal cells that are recruited to the site of the bone injury to promote blood vessel formation. Because of its primary ability to stimulate neovascularization, VEGF is of special importance in bone regeneration.[30–32] Thus, the aim of this study is to investigate the hydroxyapatite with exfoliated human deciduous tooth stem cells effect of both HA with SHED on MMP-8 and VEGF expression in the alveolar defectsdefect of Wistar rats (*Rattus norvegicus*).

MaterialsMaterial and methodsMethods

Ethical approval

The Universitas Airlangga, Faculty of Dental Medicine ethics committee granted ethical approval for both human sampling and animal experiments (171/HRECC.FODM/VIII/2017).

Study design

Design of the study

This was an experimental laboratory study with. The study used a posttestpost-test-only control group design. The In this study, the sample size was calculated using the minimal sample size formula. The sample count, which was five experimental animals in each group

(N=10, /n=5). Each group's sample was selectedpicked at random by assigning a tag number to each experimental animal and selected blindlyblind randomly.

Cell culture

The SHED was collected from deciduous teeth that met the following criteria: #83 and, #73 deciduous teeth that were free of cavities, had no root resorption confirmed by apical radiography, and had a vital and intact pulp. Healthy deciduous teeth were taken from a healthy 9-yearyears-old male, healthy child who was undergoing orthodontic treatment at the Universitas Airlangga Dental Hospital, Surabaya, Indonesia. Patient confidentiality was protected, and a signed informed consent from the patient's parents was acquired.

The SHED was isolated usingfollowing the same protocol as previously described.[33] The stemness of the SHED was confirmed by cluster of differentiation (CD) 105 (+) and CD 45 (-). The medium was changed every four days to remove the detached cell from the culture plate, and the cells were maintained for four passages. To remove debris, the cells were washed with a phosphate buffer saline.Phosphate Buffer Saline. To separate the cells and transfer them to a larger culture plate, trypsin-EDTA 0.05% was used. The SHED cells in the four passages were prepared for the next step of the investigation after they attained 70–80% confluence.[33–35] A 20-ml suspension of the SHED at passage four to five with a density of 10^6 cells waswere seeded into HAS (bio hydroxBio Hydrox hydroxyapatite, Biomaterial Center Dr. Soetomo Tissue Bank) before being placed in a 24-well tissue culture plate and prepared for the experimental group. The dose was determined using the data from a prior in vivo investigation, which reported 10^6 cells per sample.

Alveolar bone-defective animal model preparationBone Defective Animal Model Preparation

Ten healthy males, three-month-old male Wistar rats (*R. norvergicus*) of approximatelyabout 150–250 grams body weight werewas obtained from the Research Center

of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia. Five samples were randomly allocated to one of two groups: ~~Hydroxyapatite Scaffold (~~HAS~~),~~ and HAS ~~+ with exfoliated human deciduous tooth stem cells (~~SHED~~).~~

To minimize animal suffering, all experimental procedures involving animals were carried out in accordance with the National Institute~~Institutes~~ of Health's~~Health~~ Guide for the Care and Use of Laboratory Animals.[35] Because ~~of~~ the animal models originated~~came~~ from different places, they were acclimatized for a week at a temperature of 21–23°C with controlled humidity (50 ± 5%) in a 12-hour artificial light cycle (8 am to 8 pm) to let them adjust to the environment.~~same environments.~~ All of the rats were placed in polycarbonate cages sized 0.90 m ~~×~~x 0.60 m ~~×~~x 0.60 m. Furthermore, all animal models were~~model was~~ fed a regular pellet diet and given free access to water, and~~with~~ the husk was~~being~~ replaced every three days. Food consumption and fecal parameters of all animal models were routinely inspected and observed.[36] Following the induction of an alveolar bone defect by extracting the rat's mandibular incisive, samples from the ~~SHED seeded in~~ HAS and~~or~~ HAS ~~+~~ SHED groups~~alone~~ were transplanted into the affected area. ~~A~~ 5.0 suture monofilament was utilized to conduct the interrupted suture to repair the incision following transplantation.[37]

All animal models were euthanized~~terminated~~ seven days after the transplantation to analyze early alveolar bone remodeling. Euthanasia~~remodelling. Termination of animal model~~ was performed via~~done by means of~~ an overdosed rodent anesthesia, ~~with~~ an intravenous injection of 100 mg/kg BW (Pentobarbital, Pubchem, USA). This method of euthanasia was selected~~employed~~ to alleviate any~~animal~~ pain caused by the euthanization~~termination~~ process. The ~~We collected the~~ affected alveolar bone samples were collected for histological investigation following~~after~~ the animal trial~~ended~~. Using sterile sharp surgical scissors (metzenbaum scissors fine tips, no cat. 3565, Medesy, Maniago, Italy) and a tweezer (Tweezer de bakey mini, no cat. 1007/10-TO, Medesy, Maniago, Italy), the animal model's~~model's~~ head was cut from the back, exposing the anterior ~~of the~~ mandible and allowing the afflicted alveolar bone sample to be obtained. All of the animals were examined for any signs of general toxicity, such as edema or death, and their body weight was assessed ~~(~~using a digital scale (~~,~~ZB22-P,

Commented [PI3]: Should this be "mandibular incisive canal" or "incisor?"

Commented [PI4]: This is a little unclear. Did you mean, "initiate the interrupted suture's repair of the incision?"

Commented [PI5]: Did you mean something else? Death isn't a "general toxicity."

Zieis®, USA). A single blind observer performed ~~for~~ all of the measurements. Finally ~~After that~~, the affected tissue was removed and fixed in a 10% neutral buffer formalin solution.

Tissue embedding, sectioning ~~Embedding, Sectioning~~, and processing ~~Processing~~

The sample was decalcified and submerged in 10% EDTA (Ajax Finechem, Thermo Fisher Scientific, Taren Point, Australia; cat no. 17892). The samples were then processed overnight (Leica TP1020, USA) before being embedded in molten paraffin wax (Leica HistoCore Arcadia H - Heated Paraffin Embedding Station, USA). A 5 m rotary microtome (RM2235, Leica, USA) was used to cut the sections. Flattened paraffin ribbons were collected onto polysine microscope slides (Thermo Scientific) and dried at 60°C for 16 hours (Sakura Heater, Tokyo, Japan).[38]

Immunohistochemistry staining

A 3.3'-~~-~~diaminobenzidine stain kit (DAB; ~~) (~~cat no. D7304-1SET, Sigma Aldrich, US) was used for immunohistochemistry staining. This study used a 1:500 dilution of ~~vascular endothelial growth factor (~~VEGF~~)~~ antibody monoclonal (AbMo; ~~) (~~cat. no sc-7269) and ~~matrix metalloproteinases 8 (~~MMP-8~~)~~ (cat.no sc-514803; ~~) (~~Santa Cruz BiotechnologyTM, US). Using a Nikon H600L light microscope (Japan) at 400×~~400x~~ magnification, two observers manually counted and examined the number of VEGF expressions in the periodontal tissue in five ~~perspectives~~ fields of view. Each marker was~~is~~ also magnified by 1000×~~1000x~~ for context (Nikon, Japan).[38]

Statistical analysis

To analyze the data in this study, the Statistical Package for Social Science (SPSS) 20.0 version (IBM corporation, Illinois, Chicago, United States) software was utilized. A t-test ($p \leq$

<0.05) was used to compare the significant differences in VEGF and MMP-8 expressionsMMP8 expression across the groups.

Results

Result

To examine whether SHED + HAS affectedcombined with HA affects MMP-8 and VEGF expression after transplantation, immunohistochemical staining waswere performed on day 7. The number of MMP-8– expressing cells in the HA + +SHED group was significantly lower than thosethat in the HAS only group (p < <0.05; see) (Figure 1). Meanwhile, the number of VEGF-positive cells in the HA + +SHED group was significantly higher than thosethat in the control group (see Figure 2).

Discussion

Periodontitis progression is characterized by alveolar bone loss. A range of treatment techniques have beenwere proposed, including bone grafts, directed tissue regeneration, root conditioning, enamel matrix derivatives, and a combination of the above procedures. Despite this even then, the results are not unequivocal. Novel technologies based on tissue engineering (using stem cells and scaffolding) may emerge as possible therapiestherapy possibilities.[1]

In this study, the animal experiment was done in seven days to analyze the early markersmarker of alveolar bone remodeling via remodelling such as VEGF and MMP-8. This experimental work supports the idea that SHED seeded in HASHA could decrease the number of biomarker expressionsbiomarkers expression for detecting alveolar bone destruction (,such as MMP-8MMP8 expression), in bone defects after seven days when compared withto the HASHA only group. Due to their role in the pathological breakdown of the extracellular matrix (ECM) within periodontal tissues, several pieces of evidence show that the active MMP-8 (collagenase-2) derived from neutrophils isare the most critical mechanism in the tissue destruction associated with periodontal disease. Pathogens in dental plaque can trigger host

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cells to increase MMP release, which is one of the indirect causes of tissue damage that occurs in periodontitis.[39,40] A high level of MMP-8 in the HAS group~~HA-only groups~~ could be explained by an increased~~increase in~~ immune response to the presence of the scaffold as a foreign object~~item~~. A significant decrease in MMP-8 expression was noted in the HAS + ~~HA+~~SHED group compared with the HAS~~that in HA only~~ group ($p < 0.05$). This result supports the theory that the SHED as an MSCs lineage may play a role in supporting the immunomodulation towards an~~toward~~ inflammatory response suppression. Similar findings~~finding was shown~~ by Mauney et al.~~,~~ and Rahyussalim et al. showed ~~which discovered~~ that when MSCs were induced for osteogenic differentiation when the niche supported~~supports~~ the condition, their expressions of MMP-1 and MMP-8 decreased~~reduced~~. MMP-8, a collagenase that degrades collagen, will be regulated to ensure the greatest possible ECM environment and structural formation after osteogenic differentiation.[41,42]

Ceramic scaffolds~~,~~ such as HAS offer~~hydroxyapatite (HA) offers~~ the greatest promise for stem cell~~–~~-based bone engineering due to its high cell adhesion and proliferation,~~,~~[43,44] and is~~It~~ also essential in promoting SHED proliferation and differentiation.[35] Furthermore, using HA as a biodegradable scaffold provides~~scaffolds provide~~ skeletal support for osteogenic cell development during the early stages of bone repair. When SHED was seeded in an HA scaffold, the VEGF~~,~~ angiogenesis markers expressing cells significantly increased~~increase~~ compared with those in the HAS~~to that of HA only~~ group ($p < 0.05$). This could be explained by the fact that ~~as~~ HA is a porous bioceramic that permits the formation of capillaries and other blood vessels. Due to ~~form. Because of~~ their ease of vascularization and high oxygen permeability, the pores of an HA scaffold aid in osteogenesis.[45,46] SHED, in addition to its ability~~being able~~ to differentiate into osteoblasts, may also differentiate into vascular endothelial cells.[47] Angiogenesis and osteogenesis are~~have a~~ very strongly linked~~strong link~~. Angiogenesis is required to sustain and maintain bone formation and maintenance. Blood vessels also serve as a network of communication for bones and surrounding tissues.[48,49] ~~Study by~~ Cetinkaya et al. showed ~~shown~~ that VEGF expression was greatly elevated throughout the healing stage of periodontal disease.[50] Further, the ~~It was further explained in that~~ study showed that VEGF expression was ~~shown to be~~ more connected with~~to~~ the non-inflammatory component of the

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enlarged tissue than withto the inflammatory component as there was a clear positive association between the number of blood vessels and VEGF expression only in the healing group. These findings could suggest to a relationshiprelation between VEGF production and vascularization in the resolution of inflammation and the spontaneous healing of periodontal tissues.

The VEGF that expressed by osteoblasts is osteoblast important in supportingto support bone regeneration during inflammation and maintainingmaintain bone hemostasis. VEGF playsplay crucial roles in some phasesphase of the bone- remodeling process. A previousPrevious study showed that VEGF depletiondeletion in osteoblasts inhibitsosteoblast inhibit the bone- remodeling process. Macrophages,Macrophage as inflammatory cells, requirecell needs VEGF to promote the migration during inflammation phase. AdequateThe adequate VEGF levelslevel or expressionsexpression are necessary in maintaining mandatory to maintain the angiogenesis and osteogenesis in the bone- defective area.[51] In the alveolar bone- defective area, the microenvironment was hypoxichypoxia. In addition, VEGF and stem cell migration was regulated by thehypoxia condition of hypoxia. The vascularization supports bone development and theosteoblast cells proliferation of osteoblast cells.[52]

SHED showed a prominent ability to differentiate into osteogenic and odontogenic in vitro.[33] Regenerative therapy using SHED and HAHydroxyapatite can overcome the problem to regenerate alveolar- defective animal modelsmodel by increasing VEGF expression and decreasing MMP-8 expression. Compared with Comparing to Dental Pulp Stem Cells (DPSCs,), SHED showed both a higher capacity to increase osteoblast markers related tofor osteoblastic differentiation and , where, SHED expressed higher levels of ALP, Col I and OCN compared withto DPSCs.[53] The stemnessStemness and multipotency of SHED was maintained by some growth factor, such as basic fibroblast growth factor (bFGF) and VEGF.[54]

The limitations of this study were that the observationsobservation and evaluationsevaluation were performed seven days post transplantation of SHED seeded in HAS on the animal model, and only an immunohistochemical examination was performed. Further studies arewill be necessary to evaluate the changes in the alveolar bone and periodontal tissue

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post transplantation of SHED seeded in HAS~~CAS~~ in the alveolar bone defect in animal models. With a longer observation time, further studies using other methods, such as qRT-PCR ~~and~~/or the western blot analysis, could be conducted to estimate the expression of bone molecular markers. Future studies are also required to confirm the effective dose of the selected~~used~~ biomaterials when they are~~it is~~ ready to be applied in ~~the~~ clinical human studies~~study of humans~~.

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Conclusion

The expression of VEGF increases significantly with treatment of SHED seeded in HAS, whereas MMP-8 expression in the alveolar bone decreases in SHED seeded in HAS, as observed immunohistochemically.

Acknowledgements

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Disclosure

The author reports no conflicts of interest in this work.

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Figure 1. Histological sections of periodontal tissues from Wistar rats (*R. Novergicus*). (A) A positive reaction of MMP-8 in cytoplasm waswere shown in a brown color (black box) under a 400×with 400x, and 1000×1000x magnification using a light microscope following immunohistochemistry staining with antibody monoclonal (AbMo) and DAB (A). (B) The number of osteoblasts expressing MMP-8MMP8 in the alveolar bone of the rats was compared. * =*, significant between groups ($p < 0.05$).

Figure 2. Histological sections of periodontal tissues from Wistar rats (*R. Novergicus*). (A) A positive reaction of VEGF in cytoplasm waswere shown in a brown color (black box) underwith a 400×400x, and 1000×1000x magnification using a light microscope following immunohistochemistry staining with antibody monoclonal (AbMo) and DAB (A). (B) The number of osteoblasts expressing VEGF in the alveolar bone of the rats was compared. * =*, significant between groups ($p < 0.05$).

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