Calcium Carbonate of Blood Cockle (Anadara granosa) Shells induced VEGF-A Expression in Dentin Pulp Complex An In Vivo Study

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ORIGINAL ARTICLE

Calcium Carbonate of Blood Cockle (*Anadara granosa*) Shells induced VEGF-A Expression in Dentin Pulp Complex An In Vivo Study

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ABSTRACT

Introduction: Mild or moderate-intensity injury will respond with a brief inflammatory response followed by reactionary dentinogenesis. Calcium hydroxide has been considered the gold standard for pulp capping materials for decades to stimulate the formation of tertiary dentin. Blood clamshells (*Anadara granosa*) contained a high content of calcium carbonate (CaCO3) in the blood clamshells. It is a source of calcium and it has good biocompatibility to be used as a bone repair material. This study is aimed to reveal the expression VEGF-A in odontoblast pulp cells on days 1, 3, and 7 after administration of blood clam shells derived calcium carbonate in dentin pulp complex. **Methods:** Thirty Wistar rats (Rattus norvegicus) were divided into 6 groups consist of 5 random samples each. The experimental group's cavity was prepared on the occlusal side of the right upper molar, and they were treated with blood clamshell calcium carbonate suspension and sealed with RMGIC. For decalcification, the solution was replaced with ethylene-diamine tetra acetic acid (EDTA) after 24 hours and was refreshed every day. Anti-VEGF-A monoclonal antibodies were used for HE and immunohistochemical staining. **Result:** In this study, the results were obtained consecutively on the 1st, 3rd, and 7th day with p> 0.05 for VEGF-A in all groups. **Conclusion:** The application of calcium carbonate is expected to be an innovative treatment in opening up new pathways for the regenerative dentin process through upregulation of VEGF-A

Keywords: Calcium Carbonate, Dentin Pulp, VEGF-A, *Anadara granosa*, Medicine

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INTRODUCTION

Dental caries is one of the most clinical cases found in the Conservative and Endodontic Clinic of Dental Hospital, Universitas Airlangga. Raharjo reported 105 dental caries cases of 169 men (62.13%) and 146 cases of 251 women (58.17%) in 420 Rumah Sakit Gigi dan Mulut Pendidikan Fakultas Kedokteran Gigi (RSGMP FKG) Universitas Airlangga patients (1). Pulpitis reversible is a clinical diagnosis of a mild to moderate pulp inflammation caused by a stimulus, that the pulp can reverse back to its normal condition when the stimulus is removed (2). Teeth diagnosed with pulpitis reversible need a pulp protective material that has the anti-inflammation effect and good biocompatibility.
One of the treatments done in pulpitis reversible is pulp capping. Pulp capping aims to preserve the vitality of the pulp. Calcium hydroxide has been considered the gold standard for pulp capping materials for decades. Calcium hydroxide promotes the production of tertiary dentin, which can protect the pulp with the newly formed hard tissues (3), and possesses antibacterial qualities due to calcium and hydroxyl breaking down, since this hydroxyl ion has a high pH, it can kill germs in the cavity (4). However, calcium hydroxide dissolves easily in oral cavity fluid and causes a tunnel defect. The defect can raise the likelihood of marginal leakage and pulp sensitivity (5).

Blood clam (*Anadara granosa*) is an economic resource and it is easy to find in Indonesia. Blood clamshells contained of 98.7% calcium carbonate, 0.05% Mg, 0.9% Na, 0.02% P and 0,2% others. Due to high content of calcium carbonate (CaCO3) in the blood clamshells, it becomes good source of calcium that is biocompatible to be used as a bone repair material. Therefore, the blood clamshells are expected to be an alternative pulp protective material for initiating the dentin tissue deposition so that the healing process can occur by the formation of reactionary dentin. The blood

clamshells have anti-inflammatory properties as well as good stability in the wet dentine filled with intratubular fluid (6).

Mild or moderate-intensity injury triggering a brief inflammatory response followed by reactionary dentinogenesis (7). Pulps with good vascularity express endothelial growth factor A or Vascular Endothelial Growth Factor-A (VEGFA), VEGF is the main regulator of angiogenesis in pulp tissue whose changes may have a significant impact on the maintenance and regulation of pulp tissue and tooth integrity (8). VEGF is a protein specifically secreted by the endothelium, which plays an important role in angiogenesis. VEGF is the most potent angiogenic and vasculogenic factor involved in the formation of tertiary dentin. hVEGF (human Vascular Endothelial Growth Factor) has a positive effect on proliferation, differentiation, mineralization, neovascularization and production of reparative dentin from dental pulp tissue in vitro and in vivo. hVEGF has clinical therapeutic potential for the treatment of pulp disease. VEGF began to be expressed on day 3 and was maximally expressed on day 7 (9).

In the previous studies, blood clam shell extract was shown to have the highest cell bioavailability at 6.75 mg/ml and the lowest at 54 mg/ml (10). Another research states that blood clam shells derived bone graft is effective in accelerating the healing process of alveolar bones after tooth extraction (11), but there is no research yet on the role of blood clam shells derived calcium carbonate in the process of pulp inflammation. Thus, this study is expected to reveal the expression of VEGF-A in odontoblast on day 1, 3 and 7 after administration of blood clam shells derived calcium carbonate, so that blood clamshells can be considered as an alternative pulp protecting material in large carious lesions.

MATERIALS AND METHODS

The preliminary study was done to determine the amount of the blood clamshells derived calcium carbonate (powder) and aquades (liquid) to be mixed for the main research. 10 samples of blood clamshells derived calcium carbonate divided into 5 groups. Group A, the mixture ratio of calcium carbonate (powder) and aquades (liquid) is 1:1, Group B 2 : 1, Group C 3 : 1, Group D 3 : 2, and Group E 4 : 1. The blood clam shell powder and aquades were mixed and the setting time was recorded. As the result, group C which the ratio 3:1 used for the research because it has had the best result of composition.

The sample used in this study was 30 *Rattus norvegicus* strain Wistar in randomized post-test only control groups. There were treatment group and control group. The rats were male, healthy and have had weight around 300-350g. The rats were divided into 6 groups of 5 random samples each. Each of the rats was given 0,2cc/kg combine anaesthesia of Ketamine HCl and Diazepam (100mg : 10mg). Cavity preparation performed in the occlusal side of the right upper molar using 0.8 mm low-speed diamond bur (Figure 1). For the treatment group, the depth of the preparation was around 0,5-0,6 mm. The control group (Group 1-3), was treated with RM GIC (Cention N, Ivoclar Vivadent, Liechtenstein) without calcium carbonate. The treatment group (Group 4-6), were treated with blood clamshells derived calcium carbonate suspension and sealed with RM GIC. The calcium carbonate suspension was smeared into the cavity using a fine micro brush. The teeth in each group were extracted after 1, 3 and 7 days accordingly.

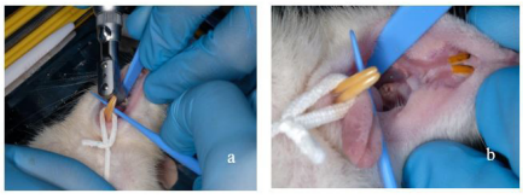

Figure 1: a. Cavity preparation using low-speed bur; b. cavity after preparation

The rats were euthanized and the right maxilla bones were removed around 12mm. Teeth were immersed in 10% formalin buffer for 24 hours, then the formalin buffer was replaced with EDTA for 60 days (renewed every day for decalcification). The paraffin block was cut by a rotary microtome as thick as 6µm.

The slides were incubated using anti-VEGFA monoclonal antibody for 60 minutes, then washed again 3 times using PBS pH 7.4 for 5 minutes. The slides were incubated using conjugated HRP for 40 minutes, then washed again 3 times using PBS pH 7.4 for 5 minutes. The slides were dripped with DAB (DiAmino Benzine) and incubated for 10 minutes, then washed again 3 times using PBS pH 7.4 for 5 minutes, then washed with aqua dest for 5 minutes. The slides were then recolored. The HE and immunohistochemical staining using anti-VEGF-A antibody monoclonal. The slides were counterstained, mounted and sealed using cover glass and observed under a light microscope.

RESULTS

Figure 2 shows the Hematoxylin and eosin staining results of the 2 groups. Anti-VEGF-A antibody monoclonal results of both control and treatment groups is shown in Figure 3. The mean expression of VEGF-A is shown in the Table I and independent t test for VEGF-A is shown in Table II.

Based on the normality test data for Shapiro Wilk for VEGF-A, both the control group and CaCO3 treated group on days 1, 3, and 7 obtained p> 0.05. Likewise, the VEGF-A expression data normality test shows p> 0.05 for all control and CaCO3 treatment groups, so

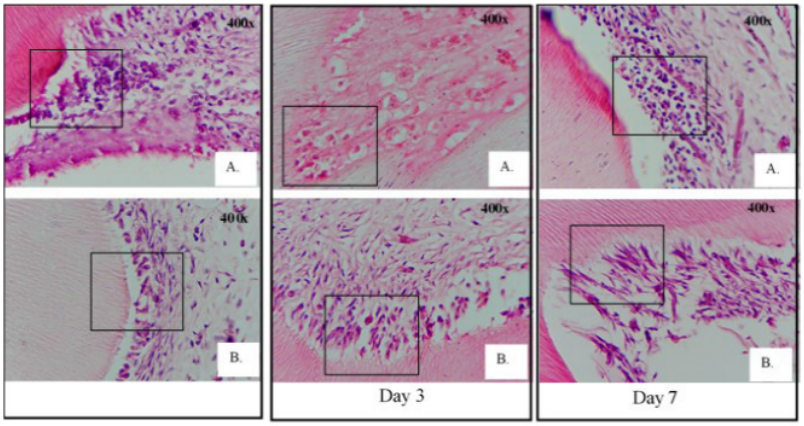

Figure 2: Hematoxylin and eosin staining results on control groups (A) and treatment groups (B)

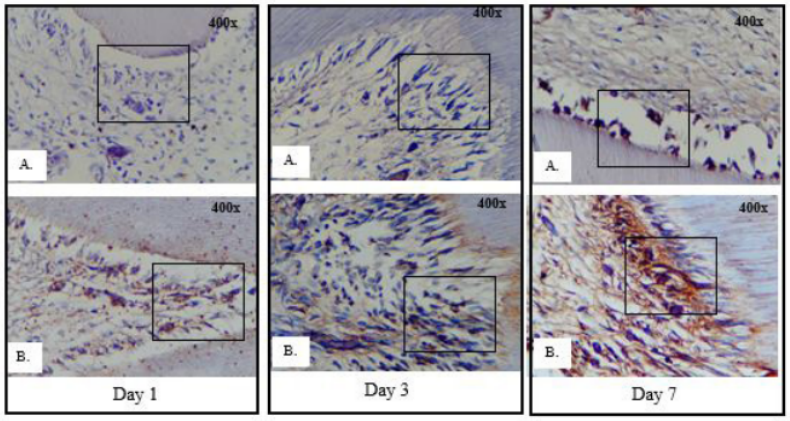

Figure 3: Anti-VEGF-A antibody monoclonal results on control groups (A) and treatment groups (B)

Table I: The mean expression of VEGF-A

Expressions	Variable	n	Mean ± SD	Min - Max
VEGF-A	Control (+) Day 1	5	3,80 ± 1,304	2 - 5
	Control (+) Day 3	5	5,60 ± 1,517	4 - 7
	Control (+) Day 7	5	9,00 ± 1,581	8 - 11
	$CaCO_3$ Day 1	5	10,00 ± 1,000	9 - 11
	$CaCO_3$ Day 3	5	12,20 ± 1,924	10 - 15
	$CaCO_3$ Day 7	5	12,80 ± 2,168	10 - 15

Table II: Effect of Calcium Carbonate from Blood Clam Shells to VEGF-A expression

Observation time	Treatment Group	N	Normality test	Homogeneity Test	*Independent t test*
Day 1	Control (+)	5	0,421*	0,489*	0.000**
	$CaCO_3$	5	0,119*		
Day 3	Control (+)	5	0,086*	0,767*	0.000**
	$CaCO_3$	5	0,928*		
Day 7	Control (+)	5	0,967*	0,222*	0.013**
	$CaCO_3$	5	0,272*		

(*) p> 0.05; (**) p <0.01

it can be concluded that all of these data are normally distributed, and it means the data have a significant difference. Furthermore, the homogeneity test was carried out using the Levene test to determine whether the data variants were homogeneous or heterogeneous. Based on the results of the Levene test, the data was considered homogeneous if p> 0.05. In this study, the results obtained consecutively on the 1st, 3rd and 7th day of treatment with p> 0.05 for VEGF-A expression, which indicates that the data is homogeneous.

In the VEGF-A group, the independent t test was carried out in the control and CaCO3 groups. From the table above, it is found that there is a significant difference in the increase in VEGF-A expression (p <0.05) between the CaCO3 and control groups on day 1, 3, and 7. This shows that VEGF-A expression was more significant in the CaCO3 treatment groups on days 1, 3 and 7 compared to control group.

Table III shows the normality test results have a p value> 0.05 at all observation times for the control and treatment groups on the expression VEGF-A. The data is normally distributed, it means the data have a significant difference. The homogeneity test results also have a p value> 0.05 at all observation times for the control and treatment groups on VEGF-A expressions. This means that the variants of the data population groups are the same. Furthermore, the post hoc test was carried out to determine the significance between groups of observation time on days 1, 3 and 7. To find out the difference with a significance level of p = 0.05 and to compare all the mean values between the tested groups, the Tukey HSD Test was used. Tukey HSD results of VEGF-A expression in control and CaCO3 groups was shown on the Table IV.

Table III: Effect of Time of Observation on VEGF-A Expression

Groups	Observation time	N	Normality test	Homogeneity Test	ANOVA
Control (+)	Day 1	5	0,421*		
	Day 3	5	0.086*	0.844*	0.000**
	Day 7	5	0.967*		
$CaCO_3$	Day 1	5	0.119*		
	Day 3	5	0.928*	0.140*	0.065
	Day 7	5	0.272*		

(*) p> 0.05; (**) p <0.01

The control group there was no significant difference in the VEGF-A expression on day 1 compared to day 3 where the p value> 0.05. While the comparison of day 3 and 7 and day 1 and 7 there is a significant difference in the control group with p value, respectively, p = 0.009 and p = 0.000. While the results of the CaCO3 group in table 6 based on the ANOVA test, there is no significant difference with p value> 0.05 between the observation groups on the 1st, 3rd, and 7th day.

DISCUSSION

The effect of application of calcium carbonate (CaCO3) shells of blood clams on VEGF-A expression on days 1, 3 and 7

Based on the result, the VEGF-A expression was significantly more in the calcium carbonate treatment groups on days 1, 3 and 7. Research from Poli reported The higher increase in VEGF-A expression in the CaCO3 group compared to the control group according was due to the calcium content of calcium carbonate found in blood clams. With the addition of calcium carbonate in the cavity that has been prepared in the treatment group, the release of Ca2+ ions will occur so that the intracellular influx (flow) of Ca2+ ions increase and causes the release of Ca2+ ions on the surface of the endoplasmic reticulum (12).

VEGF will stimulate the proliferation and activation of Alkaline Phosphatase (ALP). Ca2+ ion can also increase Alkaline Phosphatase (ALP) by activating Pyrophosphatase. Pyrophosphatase is an enzyme that catalyzes the conversion of one pyrophosphate ion to two phosphate ions which function in calcium absorption and bone formation (13).

On the other hand, the CO32- ion from calcium carbonate will react with the phosphate which comes from the hydroxyapatite in the dentin to form CO3AP compounds (carbonate apatite) and increase ALP. This is in line with the research of Setyaningrum which states that there is an increase in ALP in osteoblasts grown in apatite carbonate (14). Furthermore, ALP will activate Dentin Matrix Protein 1 (DMP1). DMP1 initiates and modulates collagen mineralization so that it initiates odontoblasts to differentiate and form mineralization in reactionary dentin more in the treatment group than in the positive control group (12).

Table IV: Tukey HSD results of VEGF-A Expression in Control and $CaCO_3$ Treated groups

Groups	Control day 1	Control day 3	Control day 7	$CaCO_3$ day 1	$CaCO_3$ day 3	$CaCO_3$ day 7
Control day 1		0,172	0,000*			
Control day 3	0,172		0,009*			
Control day 7	0,000*	0,009*				
$CaCO_3$ day 1					0,163	0,067
$CaCO_3$ day 3				0,163		0,855
$CaCO_3$ day 7				0,067	0,855	

The highest mineral in blood clam shells is Calcium so that it can strengthen teeth and bones. Blood clam shells are also used in the synthesis of nano hydroxyapatite as bone implants for bone damage because there is a very high calcium content of 66.70%. Calcium carbonate has a pH of 8-9 so it doesn't cause damage caused by high pH. Calcium carbonate has covalent bonds and has the same characteristics of electron pairs so that the bond is stable and does not dissolve easily (15).

Calcium is one of the second messengers to mediate cellular responses to various stimuli such as cell proliferation, movement, secretion and neurotransmission. Calcium plays a role in cell survival through regulation of gene expression and triggers physiological and pathophysiological apoptosis. The importance of calcium is because there are many molecules and subcellular structures involved. One of how calcium enters cells is through diffusion through calcium ion (Ca2+) channels on the plasma membrane which open and close randomly through calcium diffusion. In diffusion there is a movement of ions from high concentrations to low concentrations so that calcium ions flow through open channels with different concentrations (10).

Blood clam shells contain 100% calcium carbonate with the chemical formula CaCO3. Calcium carbonate synthesized by hydrothermal process obtained 72% hydroxyapatite (HA) content with chemical formula Ca10(PO4)6(OH)2 and 21% tricalcium phosphate (TCP) with chemical formula (Ca3PO42) (16). Hydroxyapatite in the presence of ions other than calcium, phosphate, and hydroxyl ions causes the mineral that forms teeth cannot be expressed stochiometry with Hydroxyapatite because of its ions. easily released and replaced by other ions. One of the ions that can replace the hydroxyapatite constituent groups is the carbonate ion. There are two substitution of carbonate groups, namely replacing the phosphate group to produce type B apatite carbonate and replacing the carboxyl group to produce type A apatite carbonate. The carbonate group (CO32-) in CaCO3 will substitute a hydroxyapatite constituent group to produce type A carbonate apatite, so that it can help the deposition of the tooth structure and plays a role in the balancing process of demineralization and remineralization of teeth (17).

Effect of Time of Observation on Expression of VEGF-A in Control and Treatment Groups

The results of the calculation of the average expression also increased in the control and treatment groups from day 1, day 3 and continued to increase until day 7. The results of the one-way ANNOVA difference test showed that there was a significant difference in VEGF-A expression in the control group between the observation time between the 1st, 3rd, and 7th day and there was no significant difference in VEGF-A expression in the treatment group between the observation time of day to day. -1, 3rd, and 7th.

This is in line with the results of a study by Kobayashi who reported an increase in VEGF from day 1 in cases of replanted rat teeth and increased on day 3 where regulation of growth factors to increase blood and lymph neovascularization was very active. days after replantation when there is inflammation and during the tissue destruction process (18). In addition, VEGF expression starting from day 3 to day 7 in deciduous teeth treated with Adenovirus Vector Carrying-human VEGF (AdCMV-hVEGF), reported that VEGF began to be expressed on day 3 and was maximally expressed on day 7 (9).

The Tukey HSD test results for VEGF-A expression resulted in no significant difference in the control group on day 1 compared to day 3. While the comparison of days 3 and 7 as well as days 1 and 7 there are significant differences in the treatment group based on the ANOVA test, there is no significant difference between the observation groups on day 1, 3, and day. 7.

Research by Ariesdyanata reports that the angiogenesis process begins on day 5, where the angiogenesis process begins, which begins with stabilization of new blood vessels and migration of endothelial cells to injury-affected areas induced by pro-angiogenic factors such as VGEF, FGF and TGF β. Observations were made on day 7 because angiogenesis could only be observed maximally on day 7 because the blood vessel lumen had been formed and endothelial cells had migrated into the lumen. Meanwhile, the 14th day was chosen because the angiogenesis process will decrease on the 14th day because the new blood vessels have experienced inhibition and remodelling. The purpose of the inhibition process is to prevent excessive growth of blood vessels resulting in excessive scar tissue growth, while the remodelling process aims at efficient blood flow to new tissue by breaking the long branches of blood vessels into larger blood vessels and more branching a little (19).

The hypothesis of this study is to prove whether there is an increase in VEGF expression after application of calcium carbonate from blood clam shells (*Anadara granosa*) to odontoblast cells of teeth of rats on days 1, 3 and 7, so that it can support more tissue repair. Odontoblast cells are located on the surface of dentine-pulp complex, it activated dental immune system which involved upregulating NLRP3 after exposure some of pathogen injuries such us from dental material like resin agents (20). There were many reactionary dentinogenesis where the variables used in this study were VEGF expressions. The results obtained support the hypothesis that there is an increase in VEGF expression. Increased expression of VEGF stimulate proliferation. So that this calcium carbonate material can initiate dentin tissue deposition. Reactionary dentin formation through a

longer physiological repair process due to the presence of injured odontoblasts (21) and application of calcium carbonate can induced tersier dentinogenesis which could protect vitality of the pulp.

CONCLUSION

Calcium carbonate material can initiate dentin tissue deposition through generate of reactionary dentin which have a physiological repair process due to the presence of injured odontoblasts. Further research is needed on the physical and mechanical properties of calcium carbonate as an alternative material for indirect pulp capping.

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