# Effect of Encapsulation Beta Tri Calcium Phosphate (β -TCP) from the Synthesis of Anadara Granosa Shell as Pulp Capping Material against Inflammatory Cytokines IL-10 and TGF- β

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**Abstract**

Pulp capping treatment is an endodontic treatment that aims to maintain the vitality of the pulp. Beta-tricalcium phosphate (β-TCP) has a chemical composition close to the structure of bones and teeth that can stimulate odontoblast to form dentin bridge. β-TCP can be obtained from anadara granosa hydrothermal process.

This research intends to determine the effect of β-TCP encapsulation of anadara granosa clamshell against IL-10 and TGF β expression in dentin reparative formation. Thirty-six male Wistar rats were divided into six groups: control group (-), no-treatment group, CaOH group, and treatment with β-TCP. Application of pulp capping material on the M1 upper right tooth of Wistar rats that had been prepared for class I cavities. After seven days and 14 applications, the rats were euthanized, the prepared teeth were taken, and then IL-10 and TGFβ were examined.

The data were then analyzed statistically ($p<0.05$). The analyzed data showed significant differences in each group.

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## Introduction

The development of restorative materials is ongoing, aiming to produce restorative materials that have improved physical and mechanical properties and are biocompatible for clinical application. Several in vitro studies have found that placing restorative materials in dentin can be potentially toxic, thus damaging the pulp. Requirements for pulp capping materials must meet biocompatibility requirements that are acceptable to the body and will not harm pulp and soft tissues of the oral cavity. Pulp capping material should have ideal traits, such as stimulating reparative dentin formation, maintaining pulp vitality, being bactericidal or bacteriostatic, adhesive to dentin and restorative materials, resistant to pressure during restorative placement and during the restoration period, sterile, radiopaque, and providing bacteria seal[1]. Mechanical trauma to the pulp is most common during deep and extensive carious cavity cleaning[2]. According to research by Al-Hisayat[3], among 204 perforated pulpitis teeth, there were 90 teeth (44.11%) perforated due to mechanical trauma and 114 teeth (55.9%) perforated due to caries.

Minor trauma to the pulpal ceiling does not cause damage to the odontoblast cell layer; then, these cells can activate their tertiary dentin formation to protect the pulp from further damage. When the trauma is more profound and affects the odontoblast layer, it will create a dentin bridge formation process that requires the help of progenitor cells that can differentiate into odontoblasts[2].

*Pulp capping treatment* is an endodontic treatment that intends to maintain the vitality of the pulp[4]. *Pulp capping* refers to placing a thin layer of dental material on the surface of the dentin, which aims to maintain the vitality of the dental pulp. The material that has been used and is the gold standard is calcium hydroxide. These materials a lack soluble and cause cell surface death[5,6].

Several long-term studies have proven that $Ca(OH)_2$ is less adaptable to dentin, is less

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able to stimulate odontoblast differentiation consistently, is cytotoxic to cells, and a high pH causes $Ca(OH)_2$ to dissolve quickly, resulting in tunnel defects [7,1]. Some Research concluded that the failure of pulp capping with calcium hydroxide was about 5-21% within a year, and 20% of teeth show failure in the first year and 30% after two years of calcium hydroxide used. On research concluded that the failure of pulp capping with calcium hydroxide was about 15-30% within a year[8].

The research of Maden[9] and Nowicka[10] said the application of CaOH caused higher cell death/failure of pulp capping treatment compared to other materials. Within a week, the inflammatory layer will be replaced by granulation tissue with fibroblasts and blood vessels. Progenitor cells are then induced to proliferate and migrate to the wound area, where they differentiate into odontoblast-like cells capable of synthesizing proteins involved in reparative dentin formation. The origins of stem/progenitor cells are still under research. If inflammation remains in the pulp, reparative dentin development is obstructed and will be followed by pulp necrosis.

Research by Tran[11] showed the formation of reparative dentin in mechanical trauma applied by $Ca(OH)_2$, showing a tunnel defect. This porosity can eventually lead to the entry of bacteria into the pulp. The tunnel defect is formed because the $Ca(OH)_2$ material in the process of forming the dentin bridge is less than perfect because the sealing ability in the dentin wall is not optimal and become the initial cause of this failure material[12]. In addition, the dentin bridge formed is porous and does not correctly adhere to the dentin ceiling. This causes failure because it does not show a long-term biological seal against bacteria[13]. The nature of this $Ca(OH)_2$ material also has an alkaline pH of 11-12, so it can cause local necrosis of the pulp tissue around the injured site[11].

Based on the above, it is necessary to look for other alternatives to direct pulp capping materials that can be used in dentistry which must be able to optimize dentin regeneration so that the inflammatory process and the impact of necrosis can be minimized. Utilization of essential ingredients from materials can be gathered from the natural environment, such as anadara granosa shells.

Oyster shell is waste that is not commonly used. The structure of the oyster shell is generally similar to that of cancellous bone, and its mechanical properties are similar to that of bone. Based on the research of Kamba and Zakaria[14], it appears that the presence of calcium carbonate crystals ($CaCO_3$) originating from the shell has the potential to mimic the composition, structure, and properties of the shell. The oyster shell has a relatively high $CaCO_3$ mineral composition (98.7%)[15]. In this study, the anadara granola shell was treated with a hydrothermal method for 18 hours at a temperature of $200^0$C, and sintering for 3 hours at a temperature of $900^0$C resulted in HA (15%), β-TCP (79%), and $CaOH_2$ (6%)[16]. β-*TCP* has a chemical composition that is close to the structure of bones and teeth[17-18]. *Tricalcium phosphate* (TCP) is a material that is biodegradable, bioactive, and has a high solubility level compared to HA, with one of the polymorphs β-TCP which is generally used because of its osteoconductive trait[19]. β-TCP can promote bone reconstruction and provide a better barrier than calcium hydroxide in obtaining an open apex, thus providing the same repair[19,20]. In theory, the biocompatibility of TCP with a combination of calcium release can make TCP stimulate odontoblasts so that dentin bridges can form[20].

This research inspired an investigation on odontogenesis. In the process of odontogenesis, reparative dentin formation occurs because of the bruised stimulation to the pulp ceiling. Perforated pulp ceiling due to mechanical trauma will experience an inflammatory response. The inflammatory response plays an essential role in both standard and pathological healing. Soon after trauma, the innate immune system is activated in a response known as Damage Associated Molecular Pattern molecules (DAMPs). The hypoxic environment in the wound stimulates many cell types, including inflammatory cells.

Along with the influx of neutrophils, circulating monocytes enter the wound and differentiate into macrophages in the injured tissue. The macrophage is the crucial player in the regeneration process that involves phagocytosis, antigen presentation, and secretion of various cytokines, chemokines, and growth factors (GF) that protect the body from inflammation. Macrophages have several phenotypes, such as M1 and M2 (M2a, M2b, M2c). At the beginning of inflammation, M1 is

characterized by the production of many cytokines of tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), while at a later stage, M2 is found, namely with the emergence of anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-4 (IL-4) and interleukin-13 (IL-13)[21].

Calcium/calmodulin signaling regulates gene expression in osteoblasts. Applying β-*TCP* will produce $Ca^{2+}$, directly hitting macrophages and increasing Calcium-Sensing Receptors (CaSR). Extracellular $Ca^{2+}$ that increases Intracellular $Ca^{2+}$ is activated by the mediator CaSR - to start activating Phospholipase C (PLC), leading to an increase in inositol 1,4,5-trisphosphate (IP3). Calcium activates calmodulin which activates CaM Kinase II (CaMKII) and calcineurin leading to modulation of expression of member transcription factor Activator protein 1 (AP-1). The presence of AP-1 activation enhances the regeneration process stimulated by various GFs. This process will increase the number of blood vessels due to angiogenesis, an increase in BMP2, which increases the differentiation of Mesenchymal Stem Cells (MSCs) into odontoblast-like cells, and transforming growth factor (TGF-β), which induces Alkaline Phosphatase (ALP) to increase the secretion of Collagen type I (COL-1)[22,23].

Odontoblast-like cells will stimulate Runx2 through Smad 1/5/8 through Smad 4[22]. Furthermore, Runx2 induces the formation of mature osteoblasts by secreting osteopontin and osteocalcin. ALP is also formed through Smad, which prepares an alkaline atmosphere in the formed osteoid tissue, thus triggering the formation of COL-1. Osteopontin, osteocalcin, and COL-1 will soon undergo calcification, which plays a role in the dentin matrix formation, thus accelerating the reparative dentin formation[22].

Furthermore, Runx2 induces the formation of mature osteoblasts by secreting osteopontin and osteocalcin. ALP is also formed through Smad, which functions to prepare an alkaline atmosphere in the formed osteoid tissue, thus triggering the formation of COL-1. Osteopontin and osteocalcin, as well as COL-1, will soon undergo calcification which plays a role in the formation of the dentin matrix, thus accelerating the process of reparative dentin formation[22]. The use of microparticle technology as a drug delivery system in dentistry is starting to be used. One of the advantages of microparticles is the ability to penetrate the intercellular spaces that can only be penetrated by colloidal particle size [22].

Based on the background above, the researchers wanted to find out if there could be an increase in IL10 and TGF- β expressions after the administration of β-TCP.

## Materials and methods

### Preparation of Anadara granosa

Oyster shells (Anadara granosa) are boiled for 30 minutes with boiling water. Anadara granosa shell was brushed on the outside and inside using water and soap without bleach, then dried at room temperature. After that, it was ground using a mortar and pestle until the results were in the form of powder and sifted with 200 mesh to get smaller particles. Next, the hydrothermal process was applied: mixing 10 grams of Anadara granosa shell powder dissolved in 100 ml of distilled water and 6.9 grams of 0.6 M $NH_4H_2PO_4$ dissolved in 100 ml of aquadest with a magnetic stirrer for 30 minutes. The mixed solution was then transferred to the reactor. The reactor was put in an electric oven to be heated at a temperature of 200°C for 18 hours. The results were then cooled at room temperature. After that, the heated powder is first dissolved with 100 ml of distilled water on a magnetic stirrer and then washed with distilled water using filter paper. Washing was conducted repeatedly until the reaction product separated from distilled water, indicated by the pH returning to 7. This was done to remove acidic by-products. After the pH returned to 7, the last washing was conducted with methanol to limit the agglomeration of TCP particles during drying. Samples were then dried in an electric oven at 50°C for 4 hours and then sintered at 900°C for 3 hours to remove impurities and increase the crystallinity of the sample.

### Procedure for making encapsulation (β-*TCP*)

Manufacturing Method β-*TCP*-Alginate with Gelation Ionotropic Method Aerosolization Technique. 0.5 grams of sodium alginate was then dissolved in 50 ml of aqua demineralized and stirred with a magnetic stirrer to get an alginate solution. β-*TCP* of 0.5 grams is put into 50 ml of the alginate solution that has been formed and mixed until homogeneous. Solution β-*TCP*-alginate then dripped with $CaCl_2$ solution

(0.5-gram $CaCl_2$ powder in 25 ml of distilled water) until fiber shaped by continuously stirring using a magnetic stirrer for 120 minutes at a speed of 1000 rpm. After that, the suspension formed was centrifuged at a speed of 2500 rpm for 6 minutes, then the liquid was discarded to separate the microspheres from the $CaCl_2$ solution. The microspheres were filtered, and the wet microspheres were then weighed. After that, microspheres were dried by freeze drying at a temperature of -80$^{o}$C (freezing process for 30 minutes and drying process for 12 hours).

**Pre-clinical trials on experimental animals**

This research used white rat experimental animals (Rattus norvegicus strain Wistar), which is a true experimental with a random selection of animals. The samples used were 36 tails which were divided into six groups, with criteria selected were adult males aged 4-5 months, body weight 250-300 grams, the characteristics of clear eyes characterize physical health, shiny fur, agile movements, and good or not soft faces and there was no caries in the maxillary first molar. This research was conducted upon approval from the veterinary ethics committee of the Dentistry Faculty of Airlangga University, Surabaya.

The acclimatization procedure in this research was conducted for seven days before rats were randomly chosen and divided into six groups: negative control group (K(-)-1) with seven days of treatment, negative control group (K(-)-2) with 14 days treatment, positive control group (K(+)-1) with seven days treatment, positive control group (K (+)-2) with 14 days treatment, treatment group (P-1) with seven days treatment, treatment group (P-2) with 14 days treatment.

In the negative control group, the upper first molars were prepared and filled with Glass Ionomer Cement. In contrast, the positive control group conducted direct pulp capping using the application of CaOH + aquadest in a ratio of 1:1 and filled with Glass Ionomer Cement, while the treatment group conducted direct pulp capping with encapsulation applications. β-*TCP* from anadara granosa shell + aquadest in a ratio of 1:1 and filled with Glass Ionomer Cement.

On the first day of the experiment, all groups of experimental animals were prepared for class I preparation with a low-speed round bur (0.84 mm diameter) to reach the right maxillary M1 pulp chamber. Before preparation, rats were anesthetized using ketamine HCL and xylazine at a dose of 0.11 mL/100g body weight intramuscularly.

On the 7th and 14th days, experimental animals from each group (6 groups) were sacrificed under anesthesia using ketamine-acepromazine with a dose ratio of 0.1 ml: 0.1 ml, then decapitated by taking the maxillary bone along with the left upper three molars jaw approximately ±3 cm, then put in a 10% formaldehyde buffer solution in order to prevent the tissue from breaking down, harden the tissue and increase the tissue affinity against the paint. After the tissue fixation process, the decalcification process was conducted using EDTA for two months.

After the tooth tissue is soft enough, it is processed to become paraffin blocks through the dehydration stage, clearing, impregnation, and embedding. After that, the tooth tissue in paraffin was cut using a microtome, with a tissue slice thickness of 0.5 cm, followed by staining with hematoxylin-eosin to get macrophages, and then prepared to examine the expression of cytokines using the kit: IL-10 and TGF-β. Expression was viewed under a light binocular microscope (Olympus brand) at 400x magnification. For macrophages that express cytokines, cells brown ones were counted as positive.

The data gathered were analyzed to get an overview of the distribution and a summary to clarify the results. Then, the hypothesis was tested using the One-way ANOVA parametric statistical test, followed by the LSD test.

## Results

Results of the average description showed that the group with the highest IL-10 expression was in the treatment group that applied -TCP of Anadara granosa shell on day 14, while the lowest was in the negative control group on day 7. The increase in IL-10 expression was seen on day 14, higher than on day 7 in each treatment group. On the seventh day, the highest -TCP treatment group compared with the positive control group (CaOH) and the negative control group on day 14.

| Treatment | IL-10 | | | | p |
|---|---|---|---|---|---|
| | 7th day | | 14th day | | |
| | n | x̄±SD | n | x̄±SD | |
| Control (-) | 6 | 1,000±0.894a | 6 | 3.00±0.632a | 0.001* |
| CaOH | 6 | 2,833±0.753b | 6 | 4.667±0.516b | 0.000* |
| β-TCP | 6 | 4.167±0.753c | 6 | 6.167±0.753c | 0.001* |
| p | 0.000* | | 0.000* | | |

Description: * significant at =0.05
a, b, c, superscriptthe same in one column shows no difference between groups

**Table .** Effect of administration of materials on direct pulp capping treatment against IL-10 expression.

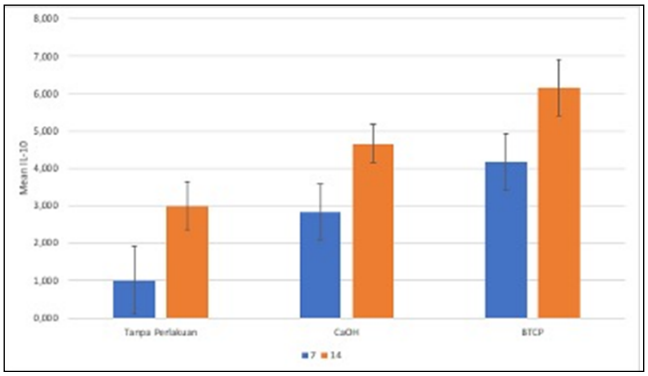


**Figure 1.** Tabel of IL-10 expression in pulp capping.

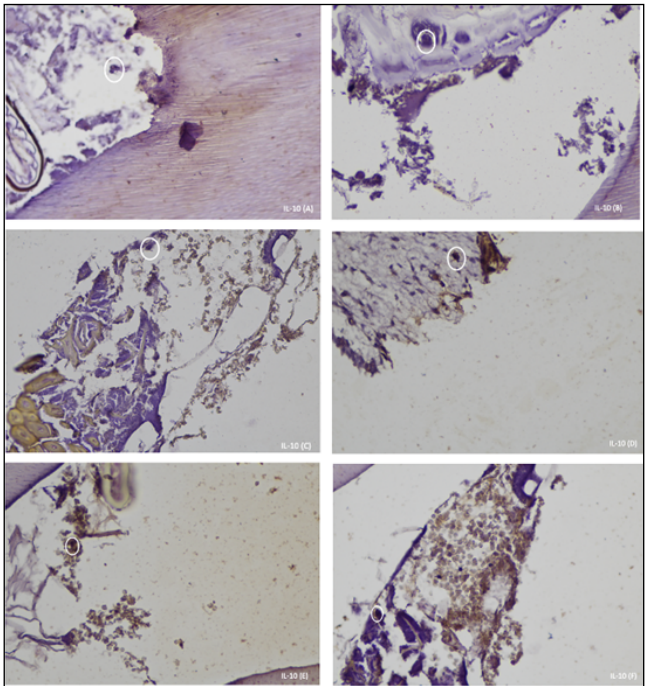

**Figure 2.** Pathological conditions where IL-10 expression was seen in macrophages found in dentin in the negative control group on day 7 (A) and 14 (B). IL-10 expression on macrophages in dentin in the CaOH group (+) on day 7 (C) and 14 (D). IL-10 expression on macrophages in dentin in the β-TCP group on day 7 (E) and day 14 (F). IHC staining with monoclonal IL-10. Observations using a light microscope at magnification of 400 times.

Data presentation from observations of IL-10 expression in macrophage cells can be seen in Table 1 and Figure 1. Pathological conditions where IL-10 expression was seen in macrophages (Figure 2)

The results of average description showed that the group with the highest TGF-ß expression was found in the treatment group that was applied to β-TCP from Anadara granosa shell on day 14, while the lowest was in the negative control group on day 7. An increase in TGF-ß expression seen on the 14th day was higher than the 7th day in each treatment group. Data presentation from observations of TGF-ß expression in macrophage cells can be seen in table 2 and Figure 3. Pathological conditions where TGF- expression was seen in macrophages (Figure 4).

| Treatment | TGFß | | | | p |
|---|---|---|---|---|---|
| | 7th day | | 14th day | | |
| | n | x̄±SD | n | x̄±SD | |
| Control (-) | 6 | 1,833±0.753a | 6 | 2,667±0.516a | 0.049* |
| CaOH | 6 | 3.167±1.169b | 6 | 4.167±0.753b | 0.109 |
| β-TCP | 6 | 4,500±0.548c | 5 | 6.167±0.753c | 0.001* |
| p | 0.000* | | 0.000* | | |

Description : * significant at =0.05
a, b, c, superscriptthe same in one column shows no difference between groups

**Table 2.** Effect of administration of materials on treatmentpulp capping indirecton TGF- expression.

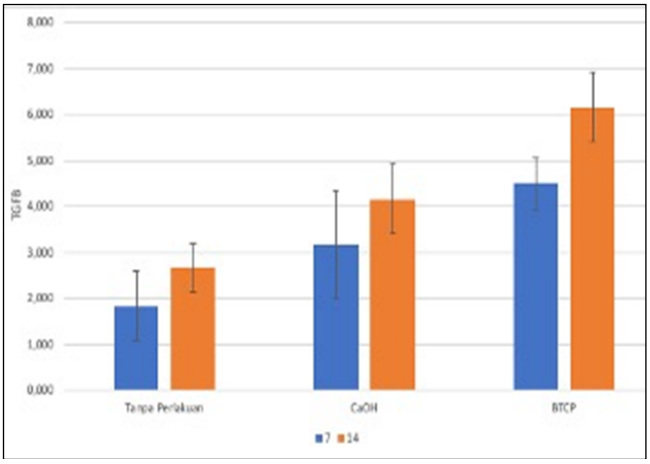


**Figure 3.** Tabel of **TGF-** expression in pulp capping.

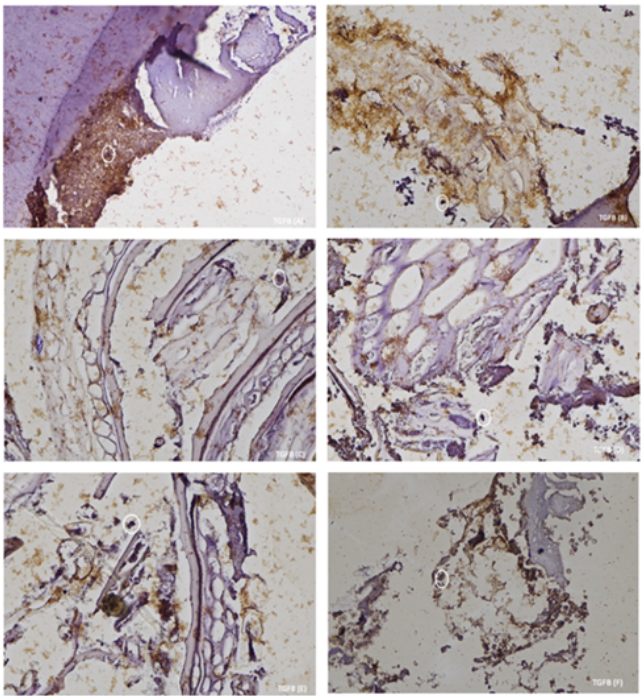

**Figure 4.** Pathological conditions in which the expression of TGF- on macrophages found in dentin in the negative control group on day 7 (A) and on day 14 (B). Expression TGF- on macrophages in dentin in the CaOH group on day 7 (C) and on day 14 (D). Expression TGF- on macrophages in dentin in the β-TCP group on day 7 (E) and on day 14 (F). IHC staining with monoclonal IL-10. Observations using a light microscope at magnification of 400 times.

## Discussion

This research was conducted to determine the effect of reparative dentin formation. On β-*TCP (beta-tricalcium phosphate)* encapsulation from the anadara granosa shell synthesis, compared with CaOH, which is currently still included in the gold standard. β-TCP was obtained from the shell of Anadara granosa that was applied with a hydrothermal process for 18 hours at a temperature of $900^0$C, sintering for 3 hours to produce HA (21%), β-TCP (79%)[16].

β-*TCP* has a close chemical composition to the structure of bones and teeth[17,18]. *β*–TCP can be used as a potential bone substitute because it is biocompatible, bioresorbable (easily absorbed), and has osteoconductivity traits[18,25]. HA and TCP can be categorized as bioceramic materials with excellent biological properties, such as non-reactivity and good osteoconductivity[1]. This research tested the cytokine expression of IL-10 and *TGF-ß*, an anti-inflammatory cytokine.

This research showed that on day 14, the group with β *-TCP* from the Anadara granosa shell application showed a significant increase in IL-10 expression compared to the other groups, while the lowest was in the untreated group (negative control) on day 7.

With the ANOVA test or difference test between groups, it was found that there were significant differences in each group, both between the 7th and 14th days in the control group (-), positive control as well as treatment. Similarly, a comparison between the $7^{th}$ and $14^{th}$ days of the same group has a significant difference.

IL-10 expression was the highest on day 14 among all groups. The inflammatory response plays an essential role in both standard and pathological healing. Immediately after trauma, the innate immune system is activated in a response known as Damage Associated Molecular Pattern molecules (DAMPs). The hypoxic environment in the wound stimulates many cell types, including inflammatory cells. Along with the influx of neutrophils, the circulating monocytes enter the wound and differentiate into macrophages in the injured tissue. Macrophages are critical players in the regeneration process that involves phagocytosis, antigen presentation, and secretion of various cytokines, chemokines, and growth factors (GF) that protect the body from inflammation. At the early inflammation, much M1, which is characterized by the production of the tumor necrosis factor-alpha (TNF-α) cytokines, interleukin-1 (IL-1), and interleukin-6 (IL-6) were found, while at a later stage the presence of M2 was found with the appearance of anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-4 (IL-4) and interleukin-13 (IL-13)[21]. Based on these findings, it is said that β-TCP can provide an excellent inflammatory response in the pulp capping treatment process. IL-10 is a robust anti-inflammatory cytokine that suppresses the synthesis of pro-inflammatory cytokines and chemokines in macrophages by activating STAT3 signaling[26]. In particular, high levels of IL-10 have been proven to be detectable in the dental pulp of teeth with deep carious lesions[27] and caries-exposed pulp[28].

*Transforming growth factor (TGF-ß) is a cytokine that plays a role in cell proliferation, cell differentiation, cell adhesion, angiogenesis, and apoptosis[29].* Transforming growth factor-β1 (TGF-β1) regulates the process of wound healing/regeneration and aging. This can be seen from the research results that there was an increase in *TGF-ß in the treatment group on day 14 compared to the other groups.*

## Conclusions

β -TCP encapsulation therapy of anadara granosa shells can be considered a pulp capping treatment product that can stimulate reparative dentin formation.

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## Declaration of Interest

The authors report no conflict of interest.

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