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RESEARCH ARTICLE

Role of Nacre and Biodentine to Inducing the TGF-β1 in the Dentin Tertiary Formation on the Pulpitis Reversible of *Rattus norvegicus*

Yeamy Agustina Marpaung[1], Trimurni Abidin[1]*, Syafruddin Ilyas[2], Irwana Nainggolan[3], Basri A. Gani[4]*

[1]Department of Dentistry Conservative, Faculty of Dentistry, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.
[3]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Sumatera Utara, Indonesia.
[4]Department of Oral Biology, Faculty of Dentistry, Universitas Syiah Kuala, Darussalam, Banda Aceh, Aceh Indonesia.
*Corresponding Author E-mail: **tri.murni@usu.ac.id and, basriunoe@unsyiah.ac.id**

ABSTRACT:

Pulpitis can cause sensitivity and trigger endodontic infections that threaten to lose dentin. Nacre and Biodentine were reported as a stimulator of TGF-β1 expression to induce tertiary dentin formation. To evaluate the ability of Nacre and Biodentine in inducing the TGF-β1 expression of tertiary dentin formation. Thirty male Wistar rats (*Rattus Novregicus*) were divided into three groups. Haemotoxylin and Eosin staining observed the dentinal bridge formation while TGF-β1 expression was evaluated by immunohistochemistry. Quantitative data were obtained based on the staining score. Kruskal-Wallis tests were used for statistical analysis**.** Nacre contains Calcium (95.04%), Oxygen (4.96%), and Carbon (0%). The dentin bridge formed after induced by Nacre and Biodentine was significant ($p<0.05$). Biodentine is stronger in reducing dentin bridges than Nacre in the hard tissue formed of the initial dentinal bridge and complete dentin bridges. The Quantity level expression of TGF-β1 of dentine is higher than that influenced by Biodentine compared to the Nacre of all score categories. They are significant among the treatment of 7 days, 14 days, and 30 days ($p<0.05$). Nacre and Biodentine can support the healing of reversible pulpitis of *Rattus novregicus*, which is indicated by an increase in TGF-β1 expression in inducing the dentinal bridge formation of 7, 14, and 30 days.

KEYWORDS: Biodentine, Dentinal bridge, Nacre, Reversible pulpitis, TGF-β1.

INTRODUCTION:

Pulpitis can trigger sensitivity due to vasodilation of blood vessels, increasing blood vessels' blood flow and permeability. Vascular responses can initiate cellular responses, causing infiltration of inflammatory cells such as neutrophils, macrophages, lymphocytes, and plasma cells around the irritation. Based on the pathophysiology, pulpitis is classified into reversible and irreversible. Reversible pulpitis is characterized by painful pain, a response to short temperatures and pain will disappear if the stimulus is removed.

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While irreversible pulpitis is characterized by spontaneous pain, this pain is still experienced even though the trigger has been released[1]. Untreated reversible pulpitis can develop into irreversible pulpitis and potentially lead to pulp necrosis[2]. This incident is known to be associated with endodontic infection, so it requires treatment to prevent dentin loss, which aims to maintain tooth integrity.

Endodontic treatment can result in a state of healing and repair or failure. Treatment of teeth with open pulp is performed for the reparative process of dentinogenesis[3]. The dentinogenesis process will be continuous from proliferation, differentiation, synthesis of collagen and minerals induced by several morphogens such as TGF-β1 and TGF-β3, BMP-2 and BMP-7 as initiators of tissue formation[4]. Besides, calcium hydroxide

(Ca(OH)2) is reported as the gold standard for pulp capping treatments. This material's weakness is not inherent in dentin. Its tissue response is low besides causing tunnel defects that allow the penetration of bacteria that will irritate the pulp tissue and cause necrosis[5]. Furthermore, pulp treatment is developed using a calcium-enriched mixture (CEM), producing hydroxyapatite crystals from endogenous and exogenous ion sources. Still, this method has a low elongation phase, affecting the pulp protection period from bacterial infections[2].

The principle of using natural ingredients in the dentinogenesis process must be immunotolerant and biocompatible[6]. Nacre and Biodentine contain calcium carbonate (CaCO3) and are biocompatible, biodegradable, osteogenic, and enamel remineralization. It is considered in the dentinogenesis process[7,8]. Nowadays, Nacre is often used as an implant material or a composite material combined with polymers or other composites for application in orthopedics and tissue regeneration[9]. Moreover, the Biodentine is a new bioactive cement with mechanical properties similar to dentin and can be used as a substitute for dentin in dental crowns and root canals[10]. Previous studies have not fully reported the expression of transforming growth factor-beta (TGF-β1) involved in reversible pulpitis therapy. This study specifically evaluated the role of Nacre and biodentine in the dentinogenesis process involving the non-specific immune system by increasing the regulator of TGF-β1 in dentin tertiary formation on the pulpitis reversible of *Rattus norvegicus*.

MATERIAL AND METHODS:

The Ethics Committee recommended this study for Experimental Animals Research-Faculty of Mathematics and Natural Sciences, University of North Sumatra, Medan Indonesia through Animal Research Ethics Committees (AREC) No.0083/KEPH-FMIPA/2019. The sample consisted of 30 teeth obtained from 10 *Rattus novergicus*, then divided into four groups: 7 days (10 teeth), 14 days (10 teeth), and 30 days (10 teeth).

Assay Material:

This study used Nacre (Infinycal, Bandung, Indonesia) and Biodentine® (Septodont, Chicago, USA). The two test materials are stored at the Chemical Laboratory, Faculty of Mathematics and Natural Sciences, University of North Sumatra, Medan Indonesia. The treatment of experimental animals in this study used the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines released by the National Center for the Replacement, Refinement, and Reduction of Animals in Research (NC3Rs) organization[11].

SEM-EDX Assay:

The profile of chemical elements and morphology of Nacre (Infinycal, Bandung, Indonesia) assayed by SEM-EDX (Scanning Electron Microscopy-Energy Dispersive X-Ray Spectroscopy), (Hitachi TM 3000, Japan)[12]. The sample is put into a vacuum in SEM, magnifying 100x dan 1000x. The EDX analysis of the composition of the Nacre elements compared to the control group.

Pulpitis Reversibel In-Vivo Model:

He (2017) adopted the reversible pulpitis model[13], the research with some modifications by researchers. Wistar rats were anesthetized Intra Musculus (IM) with 0.7mL of ketamine HCL/BB. A dental explorer disinfected the oral cavity with iodine on the maxillary first molars' occlusal surface. Then, pulp injury-induced: Preparation of maxillary molar teeth (1st and 2nd molar, left 1st molar) by making class 1 cavity 2mm wide, the cavity depth reaches the pulp roof using small diamond bur low-speed size 009 (Edenta, Switzerland). Thus pulp is exposed. Existing blood is cleaned with sterile wet cotton pellets.

The procedure refers to the Careddu study (2018)[14]. The Nacre and Biodentine were carried out on left molar 1, right molar 1, and control on right molar 2. Nacre and Biodentine powders were formulated at 700 mg and 0.20 mL, respectively (CaCl and Polymer hydrosoluble), then stirred for 30 seconds with a triturator amalgam until covering all parts of the exposed pulp using the MAP one system and waited for 15 min. The RM GIC was then mixed according to the manufacturer's instructions and placed on Nacre paste and Biodentine that had been adapted in the cavity by using a ball-pointed applicator until it filled the entire niche. Then proceed with Light curing for 40 seconds. The *Rattus novergicus* were given a soft and analgesic diet and were euthanized with Eutha 4 mL/10 lb (0.1 mL/1gram) based on groups of 7 days, 14 days, and 30 days. Then a decapitation process was carried out on the Wistar rats to separate the head from the body.

Hematoxylin and Eosin Staining:

The staining procedure Haematoxylin (H) and Eosin (E) refers to Afroze (2018)[15]. Decapitated *Rattus novergicus* are are then applied in a peroxide block, incubated for 10-15 min, and then rinsed three times with Phosphate buffer saline (PBS). Each maxilla jaw was immersed in 10% formalin for 48 h. The tooth was separated from the maxilla and engaged in a 10% EDTA solution for decalcification. The tissue was refixed with formalin buffer solution for 18-24 h. Then put into the Automatic Tissue Processor (Leica TP 1020). Secondary fixation with 10% (1) and (2) formalin buffer for 1 hour. The dehydration process used 70% alcohol, 80%, 96%, each for 90 min. Then dehydration was repeated by absolute

alcohol (1), (2), and (3) for 60 min each, then continued by purification using xylene (1), (2), and (3) each for 60 min. They were subsequently embedded with liquid 56°C (1) and (2) paraffin (Merck) for 120 min, respectively. Then it is blocked on Cessete and cooled with Parafin phase at 4˚C. Next, the samples were cut with a four μm microtome (Leica). Serial section of paraffin blocks to obtain two preparations for HE staining slides and IHC staining slides, then put into the water bath and placed on a glass object that has been smeared with glycerin. Deparaffinised in xylol (1), (2), and (3) for 15 min, respectively rehydration with 96% alcohol, 80%, and 50% for 15 min and cleaning preparations with tap water for 10-15 min.

The staining of H and E begins with preparing Hematoxillin Mayer's for 5 min. Then rinse with tap water for 5 min. Furthermore, preparations were immersed in eosin for 5 min and rinsed with tap water for 5 min. Then, the practices were put into xylol three times each for 3 min in a different container. The last step is mounting using a liquid solution then covered with a glass cover. The stained slides were examined under a microscope. Determination of qualification scores is as follows: Score 0: no dentin bridge; Score 1: No more than half of the hard tissue formed in the open area/ formation of the initial dentinal bridge; Score 2: More than half of hard tissue is included in the construction of empty/ incomplete dentin bridges; Score 3: Formed hard tissue covering all open areas/ formation of complete dentin bridges.

Immunohistochemical of TGF-β1:

Immunohistochemical (IHC) staining was carried out based on research methods conducted by Chisini (2016)[16]. The prepared slides are then applied to the peroxide block and incubated for 10-15 min, then rinsed three times with PBS. Then superblock (AAA) was used and set for 5 min. Re-rinsed three times with PBS. Subsequently, dripped antibody (TGF-β1) to taste, then incubated for 60 min and rinsed three times with PBS. Next, CRF ™ Anti-polyvalent HRP polymer was applied and set for 30 min at room temperature and washed three times with PBS. Then DAB chromagen was used, incubated for 5 minutes, and rinsed three times with PBS. Hematoxylin (HMM) was applied and incubated for 5 minutes, then rinsed three times with PBS. In the final stage, Bluing Reagent (BRT) was used and set for five sc and rinsed in tap water, dehydrated with xylene, and covered with a cover glass. Immunohistochemical analysis was carried out by microscopy observation to every stained-slides with the assessment guidelines used as follows: Score 0: no staining (no brown stain appear)/ no expression; Score 1: weakly (weakly brown color)/ weakly expression; Score 2: moderately (moderate brown color)/ moderate expression; Score 3: strongly (strong brown stain)/ strong indication.

Statistical Analysis:

Kruskal-Wallis analyzed the effect of Nacre and Buiodentine on dentin bridge formation. The differences between Nacre and Biodentine were analyzed by T-test as well. In contrast, Friedman and Wilcoxon were used to assess time's effect on the degree of dentin bridge formation and TGF-B1 expression with a limit of significance ($p<0.05$).

RESULTS AND DISCUSSION:

Based on the examination of Energy Dispersive Analysis (EDS), it was shown that Nacre contained Calcium (95.04%), Oxygen (4.96%), and Carbon (0%). This data was analyzed by the EDS system based on the Nacre's morphology at 50x magnification (Fig 1). From the results of this examination, it can be seen that calcium-rich Nacre powder is derived from the content of calcium carbonate ($CaCO_3$), which can be a reference to stimulate the expression of TGF-β1 to form tertiary dentin.

Fig 2 shows that at score 0, there is no dentin bridge, meaning at this stage, TGF-β1 is unable to induce tertiary dentin in the case of reversible pulpitis. Scores 1, 2, and 3 have shown dentin bridges' existence with different amounts in the treatment group (Table 1). Based on statistical analysis, it was shown that the appearance of dentin bridges in the Nacre, biodentine, control groups in 7 days ($p>0.05$; 0.197); 14 days group ($p<0.05$; 0.010), and 30 days group ($p<0.05$; 0.000). In general, the distribution of dentin bridge appearance on H and E staining is Score 0 (Nacre:17%, Biodentine: 13%, Control 70%); Score 1 (Nacre: 35%, Biodentine: 42%, Control 23%); Score 2 (Nacre: 30%, Biodentine: 37%, Control 0%); Score 3 (Nacre: 15%, Biodentine: 18%, Control 0%). In general, score 1 dominates the percentage of the initiation of dentin bridge formation. Nonetheless, Biodentin has a better induction power against tertiary dentin formation than the Nacre group and shows insignificant. The intensity of dentin bridge formation between groups has different effects as TGF-β1 immunostimulation induces tertiary dentin formation.

Fig 3 shows the TGFβ1 expression in scores 1, 2, and 3. Each score expression's strength varied between treatment groups seven days, 14 days, and 30 days (Table 2). Based on statistical analysis, it was shown that TGF-β1 expression in the Nacre, biodentine, and Control Group in 7 days ($p> 0.05$; 0.197); 14 days ($p>0.05$; 0.197), and 30 days ($P<0.05$; 0.000). Its means that changes in TGF-β1 expression have significant differences in the 30 days group with varying quantities. In general, the distribution of TGF-β1 expression through IHC examination was respectively: Score 1

(Nacre:17%, Biodentine:13%, Control 70%); Score 2 (Nacre:35%, Biodentine:42%, Control 68%); Score 3 (Nacre:28%, Biodentine:30%, Control 0%). On the 30th day, the biodentine group had a better ability to modulate TGF-β1 expression. However, Nacre also had a quantity similar to biodentine to induce tertiary dentin formation in irreversible pulpitis. Thus it can be explained that all treatments have a strong role to support the healing process and repair of pulp tissue based on criteria: Score 0 (No expression); Score 1 (Low expression); Score 2 (Moderate expression); Score 3 (Strong Expression).

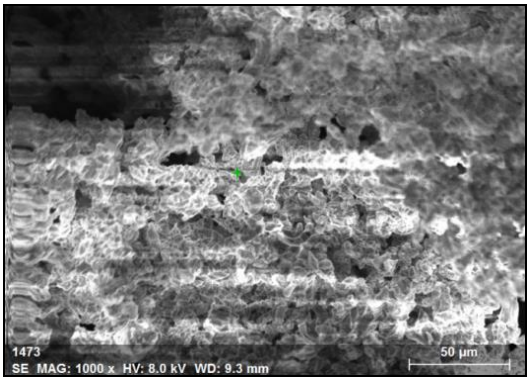

Fig. 1: Profile of Nacre morphology observed by SEM-EDS at 100x magnification

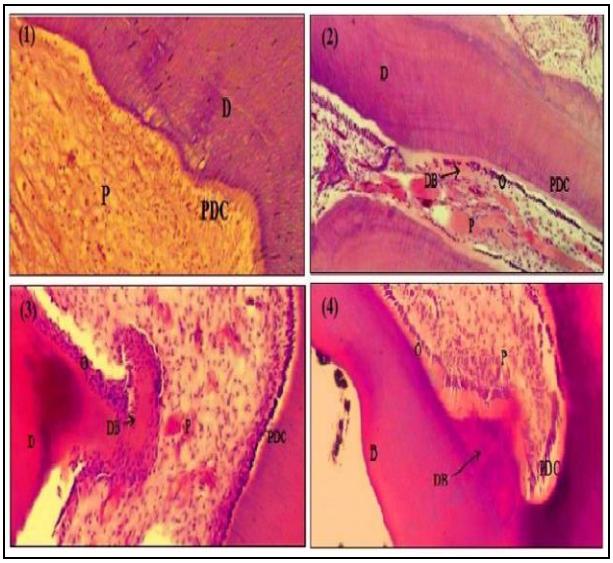

Fig. 2: H and E staining profiles of molar dentin bridges with reversible pulpitis based on the score criteria. (1) score 0; (2) Score 1; (3) Score 2; (4) score 3. D (Dentin), PDC (Pulpodentinal Complex), P (Pulpa), and DB (Dentin Bridge). Observation using stereomicroscopes, magnification of 400 x. Score 0: no dentin bridge, Score 1: No more than half of the hard tissue formed in the open area / initial dentin bridge formation, Score 2: More than half of the hard tissue formed in the open area / incomplete dentin bridge formation, Score 3: Hard tissue is formed which covers all open areas / complete dentin bridge formation

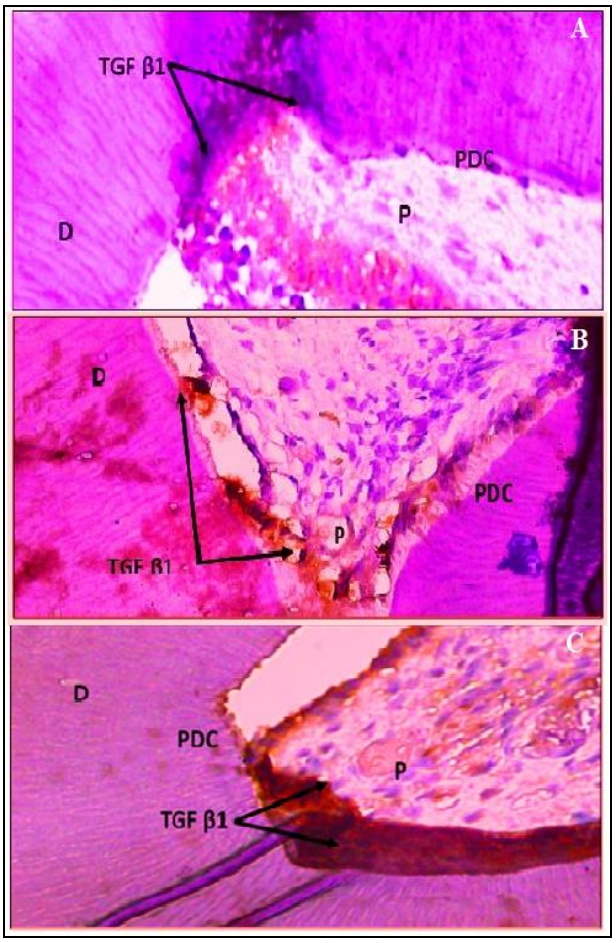

Fig. 3: Immunohistochemical Profile of TGF-β1 expression of a molar with reversible pulpitis based on score. (A) Score 1; (B) Score 2; (C) score 3. D (Dentin), PDC (Pulpodentinal Complex), P (Pulpa), and transforming growth factor-beta 1 (TGF-β1). Magnification of 400x.

This study evaluates the potential of Nacre as a natural material that has the characteristic of TGF-β1 immunostimulatory to induce tertiary dentin production in reversible pulpitis. TGF-β1 is reported as a multi-function regulator in various cellular functions, such as cell proliferation, differentiation, and matrix synthesis. Besides its role in the immune response, TGF-β1 also works in the process of repairing pulp tissue[17]. TGF-β also has implications in inducing differentiation of odontoblast cells and as a primary signal stimulator of odontoblast cells during dentinal repair[18]. EDS examination results (Fig1) show that Nacre contains calcium carbonate (CaCO3), stimulating TGF-β1 expression. In the case of reversible pulpitis, this material can help repair pulp tissue in dentin formation. Several active compounds from Nacre initiate dentinogenesis in the preparation phase of the dentin formation matrix[3].

Table 1: Distribution and frequency Score of Continuity of Dentinal bridge

Groups	7 days				14 days				30 days			
	Score 0 n(%)	Score 1 n(%)	Score 2 n(%)	Score 3 n(%)	Score 0 n(%)	Score 1 n(%)	Score 2 n(%)	Score 3 n(%)	Score 0 n(%)	Score 1 (n)	Score 2 n(%)	Score 3 n(%)
Nacre	8 (32)	2 (40)	0 (0)	0 (0)	2 (20)	7 (41)	1 (33)	0 (0)	0 (0)	1 (25)	4 (57)	5 (45)
Biodentine	7 (28)	3 (60)	0 (0)	0 (0)	1 (10)	7 (41)	2 (67)	0 (0)	0 (0)	1 (25)	3 (43)	6 (55)
Control	10 (40)	0 (0)	0 (0)	0 (0)	7 (70)	3 (18)	0 (0)	0 (0)	8 (100)	2 (50)	0 (0)	0 (0)

Score 0 (No dentinal bridge); Score 1 (formed initial dentin bridge); Score 2 (Formed Incomplete bridge/partial; Score 3 (Formed complet dentin bridge)

Table 2: Distribution and frequency of Score of TGFβ1 expression

Groups	7 days				14 days				30 days			
	Score 0 n(%)	Score 1 n(%)	Score 2 n(%)	Score 3 n(%)	Score 0 n(%)	Score 1 n(%)	Score 2 n(%)	Score 3 n(%)	Score 0 n(%)	Score 1 (n)	Score 2 n(%)	Score 3 n(%)
Nacre	0 (0)	8 (32)	2 (40)	0 (0)	0 (0)	2 (20)	7 (41)	0 (0)	0 (0)	1 (25)	4 (57)	5 (45)
Biodentine	0 (0)	7 (28)	3 (60)	0 (0)	0 (0)	1 (10)	7 (41)	0 (0)	0 (0)	1 (25)	3 (43)	6 (55)
Control	0 (0)	10 (40)	0 (0)	0 (0)	0 (0)	7 (70)	3 (18)	0 (0)	8 (100)	2 (50)	0 (0)	0 (0)

Score 0 (No expression); Score 1 (Low expression); Score 2 (Moderate expression); Score 3 (Strong Expression)

Patelli (2004) reported a statistically significant higher expression of TGF-beta was found in the odontoblast-sub-odontoblastic layer of irreversible pulpitis specimens suggesting a role for TGF-beta 1 in the dentin repair process after pulp inflammation. This research report aligns with the research results reported in Tables 1 and 2[19].

Fig 2 shows that Nacre and biodentine have the same ability to induce the formation of dentin bridges for 30 days with different distributions in each treatment group (Table 1). Tran (2019) reports that Biodentine, MTA, and Ca (OH)2 can induce vital pulp injury after applying pulp capping material. It is indicated by the formation of dentin bridges in the pulp area after 14 days and 30 days[20]. The odontoblast cells play a role during the healing process of the pulp tissue. These cells differentiate osteodentin and fibro-dentin. Besides, odontoblast cells form dental epithelium and basement membrane to form tertiary dentin[21,22].

In this study, the dentin bridge continuity formed well after 14 days and 30 days of Nacre treatment and Biodentine material. This finding is related to the stage of osteodentin formation after 14 days of pulp capping treatment and completes dentin bridge formation after 30 days[23]. On the 7th day, almost all samples showed no dentin bridge continuity was formed. This finding was related to the dentin bridge formation process that started from day 3 to day seven after treatment, known as the proliferation stage[24]. Meanwhile, the 14th day is the stage of osteodentin formation, the subsequent tubular dentin formation after treatment[25]. Other studies report that after seven days, most of the Wistar rat dental pulp has not formed a dental bridge continuity after treatment with Biodentine, tri-antibiotic paste, and calcium hydroxide paste[26,27].

Based on the facts of this research's findings, it can be reported that Nacre can stimulate the formation of dentin bridge because it contains high calcium, which is calcium carbonate, which can play a role in inducing the formation of dentinogenesis by stimulating TGF-β1 secretion. TGF-β is reported to play a role in cellular signaling to support the process of odontoblast differentiation and dentinal matrix stimulation[28]. The TGF-β isoform in the dentinal matrix can act as an additional bioactive for growth, affecting cells' behavior in the pulp-dentin complex area [29]. Fig 3 shows that at day 30, the distribution and frequency of Biodentin significantly stimulated TGF-β1 expression than Nacre (Table 2). Still, neither Biodentin nor Nacre showed a significant difference on days seven and 14. This phenomenon indicates that Nacre has the same tendency as biodentine in stimulating TGF-β1 to induce tertiary dentin formation.

Malkondu (2014) reported that the Biodentine and Nacre mechanism could form tertiary dentin to release hydroxyl ions and calcium, allowing crystalline deposits to occur on both materials' surfaces. Both materials can initiate hydroxyapatite (HA) precipitation[30]. In general, HA contains Ca2+ions which can provide biocompatibility. This property is essential to increase tolerance with the host immune system, prevent toxicity and cross-reactions, and avoid osteoid and osteogenic effects. Specifically, HA is involved in the process of dentinogenesis[31]. Watson (2014) reported that Biodentine could modulate the TGF-β1 cell secretion in the pulp to induce reparative dentin formation[32].

CONCLUSION:

The Nacre has a lower ability than biodentine, inducing dentinal bridge formation and stimulating TGF-β1 expression. Nacre can support the healing of reversible pulpitis in Wistar rats (*Rattus novregicus*), which is indicated by an increase in TGF-β1 expression in inducing dentinal bridge formation starting from 7 14 days and 30 days. The TGF-β1 expression shows a linear

process with the formation of a dentinal bridge.

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CONFLICT OF INTEREST:

The authors declare no conflicts of interest.

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