B-DeNT
JURNAL KEDOKTERAN GIGI UNIVERSITAS BAITURRAHMAH
B-Dent: Jurnal Kedokteran Gigi Universitas Baiturrahmah Vol. 12 No 2 (Desember 2025): 120-127
e-ISSN 2654-7643
https://jurnal.unbrah.ac.id/index.php/bdent/issue/view/84
ORIGINAL RESEARCH

TRANSFORMING GROWTH FACTOR BETA-1 EXPRESSION AND CYTOTOXICITY OF COMBINATION CHITOSAN-HYDROXYAPATITE IN DIRECT PULP CAPPING

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ABSTRACT

Introduction: *Chitosan is a marine natural resource with the potential to promote pulp healing. The combination of chitosan-hydroxyapatite has the ability to stimulate fibroblast cell proliferation.* ***Aim:*** *The aim of this study was to analyze the expression of TGF-β1 and the cytotoxicity of chitosan-hydroxyapatite (CH-HA) in direct pulp capping treatment.* ***Methods:*** *The study divided 5 groups of paste: a control group, CH, HA, CH-HA $_{(50:50)}$, and CH-HA $_{(80:20)}$ groups. Each of the samples was investigated using a cytotoxicity test by the MTT assay method to analyze the percentage of viability of fibroblast cells. The experimental animals used were 30 Rattus norvegicus strain Wistar. Oclusal part of molar was perforated with the tip of explorer, then filled with GIC (group$_1$), Ca(OH)$_2$ (group$_2$), CH (group$_3$), HA (group$_4$), and CH-HA $_{(50:50)}$ (group$_5$). The mandibular bone was cut, and Immunohistochemistry was carried out to examine TGF-β1 expression on 3 days of observation.* ***Results:*** *The result of analysis percentage of viability fibroblast cells in control group, CH, CH-HA $_{(50:50)}$ and CH-HA $_{(80:20)}$ uper 50% using parameter CD_{50}. Anova test showed expression of TGF-β1 significant increased ($p \leq 0.05$) in group$_5$ comparing among five groups and there was significant differences between the groups on expression TGF-β1.* ***Conclusion:*** *The combination of CH-HA was nontoxic and it could accelerate pulp healing by increasing expression of TGF-β1.*

Keywords: *chitosan, cytotoxicity, hydroxyapatite, pulp capping, TGF-β1 expression*

Received (04/07/2025);
Accepted (31/12/2025);
Available online (07/01/2026)

DOI:
https://doi.org/10.33854/jbd.v12i2.1878

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INTRODUCTION

Chitosan (CH) is a marine natural resource with potential for dental regeneration. Chitosan is a marine polysaccharide derived from chitin, made most often from shells of crustaceans. Indonesia is an archipelagic state that is mostly covered by water. The area of Indonesian sea waters reaches 71% of the entire territory of Indonesia. Thus, Indonesia is a country that is abundant in biological natural resources. The tiger prawn (*Penaeus monodon*) from Tarakan waters, East Kalimantan, is a high-value crustacean and quality export commodity.[1] Chitosan has characteristics such as mucoadhesive, biorenewability, biodegradability, biocompatibility, bioadhesivity, antimicrobial activity, and nontoxicity.[2,3] Chitosan powder synthesized from *Penaeus monodon* shrimp shells of Tarakan waters has a high degree of deacetylation of 93%. The degree of deacetylation

is one of the characteristic indicators of chitosan powder that determines biological activity.[1]

When dental pulp tissue is exposed to caries, trauma, and mechanical activity, it becomes inflamed and considered for pulp caping therapy. Chitosan could continually release vascular endothelial growth factor (VEGF) and regulate the differentiation of odontogenic dental pulp stem cells (DPSCs), thus becoming a potential bioactive molecule in dental pulp capping therapy.[4] Chitosan has high biocompatibility, antibacterial activity, and promotes the pulp healing process. Chitosan has several limitations, such as crystalline structure, low solubility in water, and low mechanical properties. Single polymer material has poor mechanical characteristics and rapid biodegradability. The calcium phosphate compound of hydroxyapatite improves the mechanical characteristics of its combination. A combination of chitosan and hydroxyapatite (CH-HA) provides a synergistic effect to promote dental pulp regeneration.[4,5] The application of a combination of CH-HA paste is considered a direct pulp capping material healing since it increases VEGF expression, new blood vessels, and proliferation of fibroblast cells.[6] The combination of chitosan and nanohydroxyapatite significantly upregulated important growth factors such as FGF, VEGF, PDGF, BMP-7, and angiopontin-2 on the healing of injured pulp.[7]

Transforming growth factor beta 1 (TGFβ1) plays a crucial role in pulp healing. TGFβ1 regulates proliferation, migration, and pulp stem cells (DPSCs) differentiation. Previous study reported that TGFβ1 regulates differentiation of odontoblast cells through AKT, Erk1/2, and p38 MAPK signal pathways and regulates inflammatory responses. Within pulpal inflammation, TGFβ1 has a role in chemotaxis of inflammatory cells, angiogenesis activity, and mineralized tissue formation. TGFβ1 enhances pulp tissue healing.[8] Chitosan contains active N-acetyl-D-glucosamine dimer, which activates macrophages to secrete important growth factors in the repair of pulp-dentin complex, such as TGFβ1, FGF, and VEGF.[6,8] Calcium hydroxide (Ca(OH)2) has been the gold standard material used for direct pulp capping until now. Nevertheless, it has been reported that Ca(OH)2 can cause superficial coagulation necrosis and apical abnormalities.[6] In dentistry, however, the requirement for pulp capping dental material is antibacterial activity, nontoxic, noncarcinogenic, and having good biocompatibility. Therefore, biocompatibility testing is required.[6,7,9] This study aims to analyze the cytotoxicity of chitosan-hydroxyapatite (CH-HA) and expression of TGF-β1 in direct pulp capping treatment.

METHODS

Synthesis of chitosan paste and hydroxyapatite paste

Chitosan powder was synthesized from tiger prawn *(Penaeus monodon*) from Tarakan waters, East Kalimantan, Indonesia. Synthesis of chitosan powder using the Deproteinase, Depigmentation, and Deacetylation (DPA) method resulted in a deacetylation degree of powder was 93%. Three grams of CH powder dissolved in 100 mL of 2% acetic acid (Sigma-Aldrich). It was added with NaOH 1,25% (Merck) to neutralize the pH, then at a speed of 2000 rpm, it was centrifuged for 30 minutes. Pasta consistency was made by adding the

formulation with HPMC solution and 0,9% saline (Bate Chemical Co.Ltd.).[6,10]

Three grams of hydroxyapatite powder (Sigma-Aldrich, Product number: 900204) dissolved in 100 mL of 2% nitrate acid (Sigma-Aldrich). It stirred and added with HPMC solution and 0,9% saline (Bate Chemical Co.Ltd) until a paste consistency. CH-HA paste formulation was fabricated by mixing chitosan paste and hydroxyapatite paste in a ratio of 50:50, then placed on a petridisk for 24 hours.[6,10]

Cytotoxicity test

The sample was divided into five groups: control, CH, HA, CH-$HA_{(50:50)}$, and CH-$HA_{(80:20)}$ groups. Cytotoxicity test was conducted by the Methyl Thiazol Tetrazolium (MTT) Assay method. Fibroblast Baby Hamster Kindy (BHK-21) cells with a cell density of 3 x 10^3 cell/ml in Eagle's *minimum essential medium (*MEM), Kanamycin, Foetal Bovine Serum (FBS) 10%,, Fungizone 100 units/ml as culture media were placed into 96 well culture plate. Each well plate contained 100 µl cell suspension, and 50 µl of CH, HA, CH-$HA_{(50:50)}$, and CH-$HA_{(80:20)}$ paste in the treatment groups. The control group contains media culture only. The plates were incubated at 37° C for 20 hours. 5 mg/ml of thiazoly blue tetrazolium bromide MTT solution was diluted in 25 ml PBS, added to each plate, and then incubated at 37° C for 4 hours. After the last incubation period, it was replaced with 50 ml of dimethyl sulfoxide (DMSO) added to each plate and incubated at 37° C for 5 minutes. The absorbance or optical density value was analyzed at a 620 nm wavelength using an ELISA Reader. The indicator of toxicity test using the CD50 parameter, which is the percentage of viable cells above 50%.[9,10]

In vivo study

This research comprised 30 Rattus norvegicus strain Wistar with a body weight of 200- 250 grams and aged approximately 8–16 weeks. The acclimatization for 7 days was performed at a temperature of 29˚C. Research was obtained permission from the Ethical Commission of the Dentistry Faculty, Universitas Hang Tuah under registered number 022/KEPK-FKGUHT/X/2022. It is divided into 5 groups, with 6 samples in each group.

Ketamine 10% injection and Xylazine 2% injection are used for anesthesia. The preparation on occlusal part of Molar Rat using tapered round diamond bur of low speed and tip of explorer on diameter and depth of 0,1 mm to perforated the cavitas, then filled with Glass ionomer cement/GIC ($group_1$), Ca(OH)2 ($group_2$), CH ($group_3$), HA ($group_4$), and CH-$HA_{(50:50)}$ ($group_5$).[1,11] Three days after application of the paste, animals were sacrificed. Mandibular molar bone was taken and fixed with put into 10% formaldehyde buffer solution, and using Ethlylenediaminetetraacetic acid (J.T Baker MDL Number: MFCD00150037UK) to decalcify. The specimens were made in a sagittal section of 4–5 µ thickness and Immunohistochemistry with TGFβ1 monoclonal antibodies (Santa Cruz Bio Inc, [3C11]: sc-130348). After that, the expression of TGF-β1 was observed on 3 days by a light microscope (Nikon H600L microscope, Japan) at 100x,400x, and 1000x magnification.[6]

The data tabulation and statistical analysis of expression TGF-β1 were performed on normal data with the Shapiro-Wilk test and homogeneity of data using the Levene test. It was followed by a one-way analysis of Variance test (ANOVA) and a Tukey HSD test ($p<0.05$).

RESULTS

Cytotoxicity test

The result of the cytotoxicity test of the combination CH-HA on cell culture was determined by the absorbance or optical density value of the cells. It is shown in Table 1 and Figure 1.

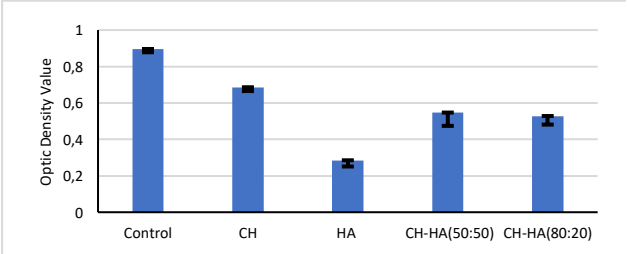
TGF-β1 Expression result

TGFβ1 expression on day 3, with immunohistochemistry staining shown in Figure 3. Based on Table 2 and Figure 2, the highest expression of TGFβ1 on pulp healing was observed in the application of CH-$HA_{50:50)}$ ($group_5$). It is followed by CH ($group_3$), HA ($group_4$), Ca(OH)2 ($group_2$) and GIC ($group_1$).

Table 1. Average optic density value and cell viability in each group

Group	Optic Density value X ± SD	Cell viability (%) X ± SD	n
Control	0,896 ± 0,018[a]	100 ± 0,00[a]	8
CH	0,686 ± 0,021[b]	77,412 ± 1,202[b]	8
HA	0,285 ± 0,034[d]	37,643 ± 2,016[d]	8
CH-$HA_{(50:50)}$	0,547 ± 0,073[c]	68,725 ± 2,211[c]	8
CH-$HA_{(80:20)}$	0,528 ± 0,047[c]	62,105 ± 1,472[c]	8

Note: [abc] different superscripts show that there were differences between groups (Tukey HSD test)



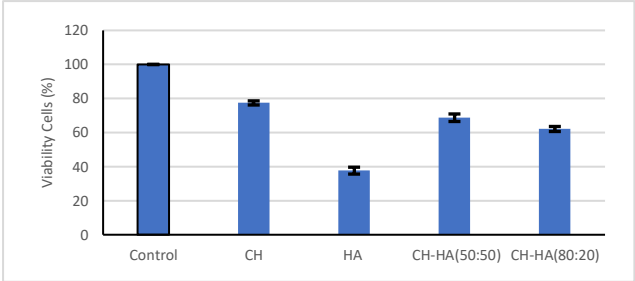


Figure 1. The graphic of optic density value and viability cell of control, CH, HA, CH-$HA_{50:50)}$ and CH-$HA_{(80:20)}$ groups.

The result of the Shapiro–Wilk analysis data was homogeneous and had a normal distribution ($p>0.05$). There was a significant difference in TGF-β1 expression in CH-$HA_{50:50)}$ ($group_5$) compared to CH ($group_3$), HA ($group_4$), GIC ($group_1$) and Ca(OH)2 ($group_2$) on day 3 observation. It showed with an ANOVA test value $p=0.00$ ($p\leq0.05$). The Tukey HSD test showed that CH-$HA_{50:50)}$ ($group_5$), CH ($group_3$), and HA ($group_4$) increased the expression of TGF-β1 in dental pulp healing treatment.

Table 2. Average TGF-β1 expression in each group

Group	TGFβ1 expression X ± SD	n
GIC (Group 1)	3,4 ± 0,7[a]	6
Ca(OH)2 (Group 2)	6,2 ± 0,9[b]	6
CH (Group 3)	8,5 ± 1,1[c]	6
HA (Group 4)	6,4 ± 0,073[b]	6
CH- $HA_{50:50}$ (Group 5)	9,4 ± 0,047[d]	6

Note: [abc] different superscripts show that there were differences between groups (Tukey HSD test)

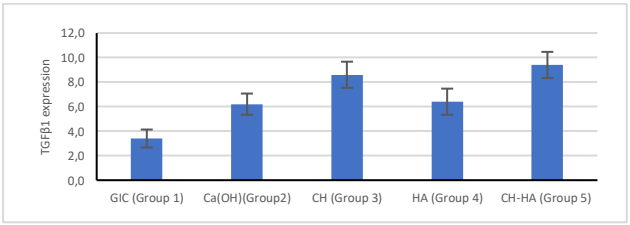


Figure 2. The graphic of TGF-β1 expression of control, Ca(OH)2, CH, HA and CH-$HA_{50:50)}$ groups

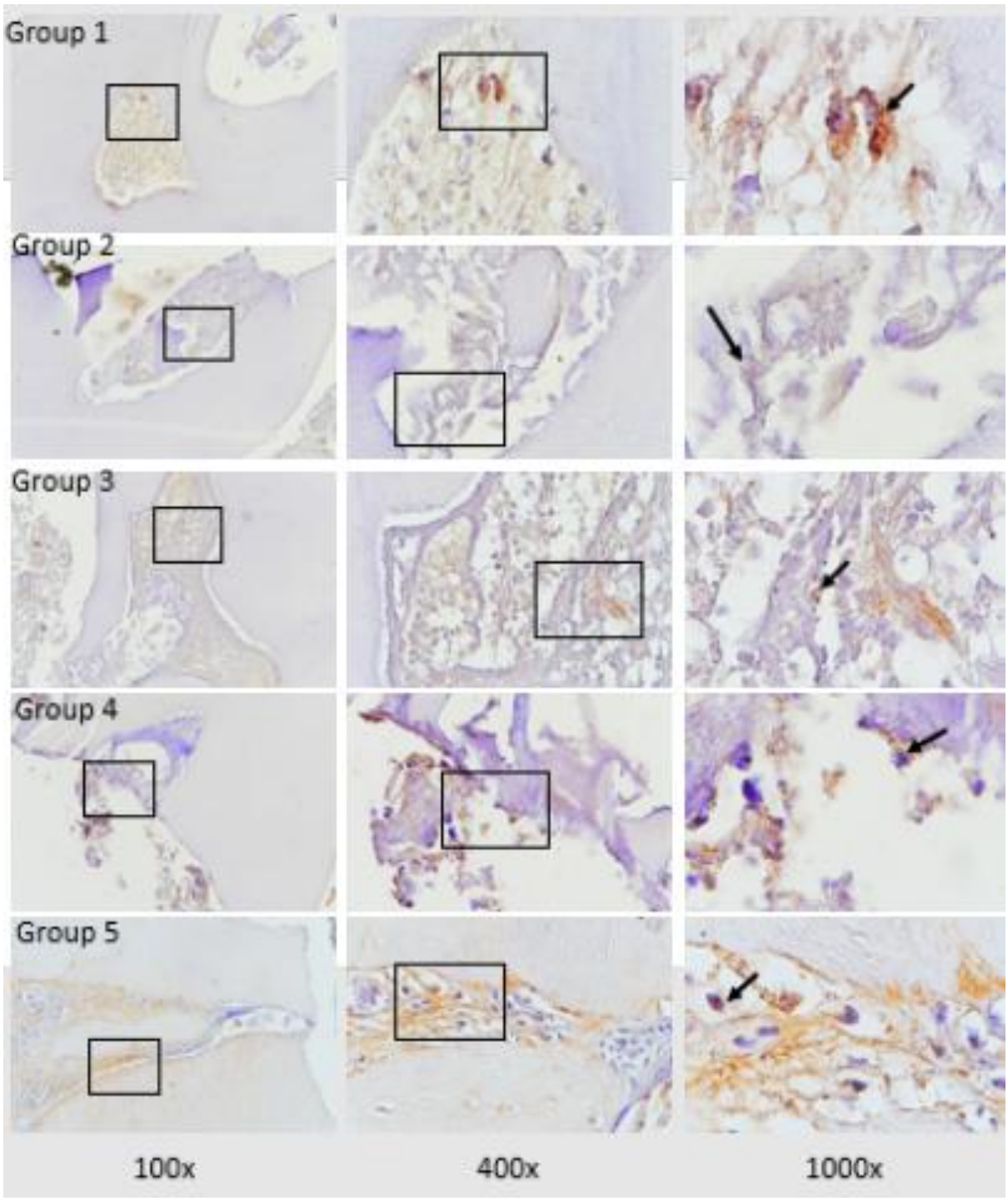


Figure 3. The expression of TGFβ1 on day 3 (black arrow). GIC ($group_1$), Ca (OH)2 ($group_2$), CH ($group_3$), HA ($group_4$) and CH-HA $_{(50:50)}$ ($group_5$) with 100x, 400x and 1000x magnification.

DISCUSSION

In this study, a descriptive analysis of the cytotoxicity test showed that the optical density values of the CH-HA group were higher than those of the HA group. The percentage of viable cells of Control, CH, CH-$HA_{(50:50)}$ and CH-$HA_{(80:20)}$ groups were uper 50% using parameter CD_{50}. The HA group was under 50%. All groups have normal distribution and homogeneity ($p>0.05$). The multiple comparison test result

with the Tukey HSD test is shown in Table 1. It shows that there is a significant difference in optic density and viability cell among control and (CH, HA and CH-HA) groups with a probability value ($p \leq 0.05$). There is no significant difference in optic density ($p=0.078$) and viability cell ($p=0.064$) among CH-$HA_{(50:50)}$ and CH-$HA_{(80:20)}$ group.

Chitosan contains N-acetyl-D-glucosamine, which is structurally similar to glycosaminoglycans (GAG). It is biocompatible and can interact with various growth factors, such as TGF-β1 and FGF-2, which initiate fibroblast cell proliferation and trigger fibroblast cell activity.[12,13,14] Chitosan made using deproteination, demineralization, depigmentation, and deacetylation methods has a high degree of deacetylation. Chitosan with a high degree of deacetylation has more amine groups (-NH2) of the chitosan molecule, so that the chitosan is more reactive and increases fibroblast proliferation. Deproteinase phase on the synthesis method of chitosan powder breaks the bond of protein and chitin, so it affects no toxicity.[15,16]

The dehydrogenase process of the MTT assay breaks the MTT ring, reducing dissolved yellow tetrazolium to an insoluble blue-purple formazan product. The changing color becomes a parameter to account for absorbance or optical density value and viability of the cell.[9,17] MTT assay measuring the cellular activity on cell metabolism.[9] Cell Viability percentage in the HA group is under 50%. Hydroxyapatite, which has calcium and phosphorus content, can create a favorable environment for the proliferation of fibroblast cells, thereby increasing the viability of fibroblast cells.[18] However, in this study, hydroxyapatite was dissolved with nitric acid without neutralizing the pH, so that it had a low pH of 2.20. Acidic media induce DNA damage and excessive intracellular ROS production in fibroblasts and lead to cell death. Nitric acid is a strong acid that easily reacts with alkalis and oxides by forming salts, which can significantly reduce the viability of fibroblast cells.[19] he Combination chitosan-hydroxyapatite paste CH-$HA_{(50:50)}$ and CH-$HA_{(80:20)}$ groups were non-toxic for BHK-21 cell culture. There was synergistic biological performance of the combination CH-HA, allowing a microenvironment for fibroblast cell viability. Combination CH-HA is required for pulp capping material to promote pulp healing.[6]

In this study, the expression of TGF-β1 in CH ($group_3$), HA ($group_4$) and CH-$HA_{50:50)}$ ($group_5$) was higher than GIC ($group_1$) and $Ca(OH)2$ ($group_2$) as the control group. There was a significant difference compared to the control groups. CH is a polycationic complex marine polysaccharide that regulates inflammatory cells such as polymorphonuclear leukocytes and macrophages. Starting on day three following the pulp exposure, macropages interact with each other in the pulp healing process. It switches from M1 polarization to M2 polarization during the late stage. CH activates macrophages to secrete growth factors such as TGF-β1, PDGF, VEGF, FGF, and BMP-2, which play a crucial role in dentin formation and pulp regeneration.[6,14,20] Some studies reported early reparative dentin formation and odontogenic differentiation of DPSC relate to TGF-β1 expression.[8]

TGF-β1 expression in HA ($group_4$) was higher than in GIC ($group_1$) and Ca(OH)2 ($group_2$). The calcium phosphate of HA could provide good osteointegration to pulp tissue. The porous material of HA could accelerate the angiogenesis process to promote tissue mineralization and dentin reparative.[22,23] The persistent acidity of glass ionomer may cause chronic inflammation and a lack of dentin formation.[24] $Ca(OH)_2$ been the standard choice material for direct pulp capping. However, the application of $Ca(OH)_2$ reported to cause superficial necrosis on exposed pulp because it releases hydroxyl ions, induces proinflammatory proteins, and an incomplete reaction of calcium and hydroxide could trigger internal root resorption and apical lesion.[12,15]

CH has good biocompatibility and antibacterial properties; however, its single polymers have low mechanical characteristics and rapid biodegradability. The combination of CH-HA has a synergistic effect to improve physical and mechanical properties.[2,23] Polycationic marine nature with N-acetyl-D-glucosamine compound of chitosan will cross-link with glycoproteins and stimulate macrophages to secrete growth factors and complement protein, promoting mineralized tissue formation.[6,23] In our study he expression of TGF-β1 in $CH\text{-}HA_{50:50)}$ ($group_5$) was higher than CH ($group_3$) and HA ($group_4$). TGF-β1 promotes early odontoblastic differentiation of dental pulp stem cells. TGF-β1 is a multifunctional cytokines which at the cellular level promote pulp healing to regulate proliferation, migration, and differentiation of odontoblast-like cells. TGF-β1 regulates not only via small mothers against Smad 2 and Smad 3 signaling, but also through pathways like mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (P13K) or protein kinase β (AKT).[8,21] During pulp healing, TGF-β1, as a signaling molecule that mediates cellular activity, could be released from the extracellular matrix and play a role in the regulation of inflammatory responses. The increase in TGF-β1 expression promotes reparative dentin formation.[8] The application of $CH\text{-}HA_{50:50)}$ paste could promote dental pulp healing by increasing TGFβ1 expression. TGFβ1 plays an important role in regulating odontoblastic differentiation of dental pulp stem cells.

CONCLUSION

It can be concluded that the combination of Ca-HA paste was nontoxic with BHK-21 cell culture, and it could accelerate dental pulp healing by increasing the expression of TGF-β1. However, further studies are needed to improve the ideal characteristics of CH-HA pasta and to explore synergistic use with bioactive molecules or growth factors so it can be considered as a potential pulp capping material.

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