Gelatin/chitosan delivery system improves stability and regenerative potential of $Ca(OH)_2$ in an open dental pulp model

Endytiastuti Endytiastuti[1], Retno Ardhani[2]*, Bidhari Pidhatika[3], Yogi A. Swasono[4], Reza P. Rudianto[5], Juni Handajani[6], Ghadah A. Al-qatta[7], Iwa S. R. Sudarso[1], and Fauzi Mh Busra[8]

ABSTRACT

Calcium hydroxide ($Ca(OH)_2$) has a long history as an agent to induce hard tissue regeneration in teeth. However, its high solubility requires inefficient repeated applications. Its alkalinity has two-sided effects: antibacterial property, but simultaneously compromises cell viability. This study prepared a composite of gelatin/chitosan to deliver $Ca(OH)_2$ using tetraethyl orthosilicate (TEOS) as the crosslinker. The chemical and physical properties of the composite were compared with unmodified $Ca(OH)_2$ aloneusing Fourier-transform infrared spectroscopy, X-ray diffraction, and scanning electron microscopy with energy-dispersive X-ray spectroscopy, followed by an investigation into the release or dissolution of Ca^{2+} from both materials. A total of 16 Wistar rats were allocated to receive either the composite or $Ca(OH)_2$ after dental pulp exposure. Regenerative potential was assessed after 7 and 14 days by histological evaluation of odontoblast-like cell numbers and transforming growth factor β1 (TGF-β1) expression, with statistical analysis performed at a 95% confidence level. The gelatin/chitosan/$Ca(OH)_2$/TEOS composite was successfully synthesized and exhibited controlled Ca^{2+} release. The results demonstrated a higher odontoblast-like cell proliferation and stronger TGF-β1 expression in the composite-treated group after 7 days of application, indicating a more intensive regeneration than the $Ca(OH)_2$ control. After 14 days, the number of odontoblast-like cells in both groups did not differ significantly. However, TGF-β1 expression was significantly more pronounced. In conclusion, the incorporation of $Ca(OH)_2$ into a gelatin/chitosan matrix using TEOS as a crosslinker successfully decreases its solubility without impairing the ability to induce dental pulp regeneration.

Keywords:
Polymer; Pulp capping; Regenerative endodontic; Tetraethyl orthosilicate; SDG 3

***Corresponding author:**
Retno Ardhani,
retnoardhani@mail.ugm.ac.id

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1. Introduction

The importance of dental health in the permanent dentition for overall human wellness is undeniable, and the long-lasting maintenance of natural teeth remains a primary goal in dentistry. However, an analysis from the Global Burden of Diseases study identified a global increase in dental caries incidence from 1990 to 2021, with young adults identified as the most affected group. The study also showed that the incidence escalated in the 5–9-year age group.[1] These findings indicate that caries is detected soon after the eruption of permanent teeth. These newly erupted permanent teeth are usually immature, characterized by an open root apex, and apical closure requires a healthy dental pulp.[2]

Caries is a chronic, multifactorial disease that is clinically observed as demineralization of dental hard tissues. The progression of demineralization, caries removal, or traumatic injury may expose the pulp tissues. In such situations, treatments are recommended to maintain the pulp tissue vitality, allowing apical closure of immature permanent teeth. Moreover, the advancement

of regenerative treatments in dentistry provides the potential for necrotic pulp tissue to regenerate and restore its natural functions.[2,3] In mature teeth, where apical closure has already formed, hard tissue formation is intentionally induced to reform the damaged dentin due to caries lesions, thereby ensuring continued protection of the dental pulp.[4]

Calcium hydroxide ($Ca(OH)_2$) has been utilized for a long time as a promoter of hard tissue growth in dentistry. It shows a high success rate, hovering around 90%. $Ca(OH)_2$ contributes hydroxyl ions to the surrounding, exerting its antibacterial effect and healing potential. It also provides calcium ions (Ca^{2+}), which are crucial for the formation of hard tissues. Due to its low dissolvability, multiple visits are required for the reapplication of $Ca(OH)_2$ until apical closure is detected. In addition, its high pH is potentially toxic to cells, thereby impairing their regenerative capacity.[2,3,5,6]

In this study, an alternative to $Ca(OH)_2$ was prepared by combining gelatin and chitosan with $Ca(OH)_2$ using tetraethyl orthosilicate (TEOS) as a crosslinker. Over the past 3 years, a gelatin/chitosan polymer has been designed as a delivery system for inorganic materials, biomolecules, or drugs with the capacity to induce tissue regeneration and eradicate infections.[7,8] Either gelatin or chitosan was applied for dental pulp regeneration, as a scaffold or delivery system, and both demonstrated a promising regenerative effect.[5,6,9] Unlike the previous studies, which incorporated carbonated apatite into the gelatin/chitosan polymer,[7,8] this study adopted the method used in Handajani *et al.*[10] and Abdulrahman *et al.*,[11] combining the polymer with $Ca(OH)_2$. The inorganic mineral $Ca(OH)_2$ was bound to polymer chains in the natural organic gelatin/chitosan polymer matrix through TEOS as a crosslinker. Adjusting the crosslink density between the inorganic mineral and organic polymer by varying the TEOS concentration controlled the release of Ca^{2+} into dental pulps. TEOS contains silicon (Si), which binds in siloxane form to form a silica core that prevents $Ca(OH)_2$ from spontaneous release.[8] A composite of gelatin/chitosan/TEOS/$Ca(OH)_2$ was synthesized, and its chemical and morphological characteristics were compared with those of unmodified $Ca(OH)_2$. In addition, the contribution of the composite to the environmental Ca^{2+} concentration was assessed to predict its role as a provider of inorganic materials for hard tissue formation.

This study further investigated whether the gelatin/chitosan/TEOS/$Ca(OH)_2$ promotes a stronger regenerative response in pulp tissue compared to unmodified $Ca(OH)_2$ in an *in vivo* open dental pulp model. Regeneration was evaluated based on the number of odontoblast-like cells and the expression of transforming growth factor β1 (TGF-β1) after 7 and 14 days of application. Both parameters were relevant to observing the regeneration of dental pulp tissue. Physiologically, odontoblast-like cells were differentiated from dental pulp stem cells (DPSCs) in response to a caries lesion, and TGF-β1 promotes the early stage of the differentiation process.[12,13]

2. Methods

2.1. Material preparation

To prepare the composite, previous methods[7,8,10,11] were modified by substituting carbonated hydroxyapatite with $Ca(OH)_2$. Briefly, 100 mL of 0.02 g/mL bovine bone gelatin hydrogel in distilled water was mixed with 100 mL of 0.02 g/mL chitosan in 0.5 M acetic acid for 1 h at 50°C. Simultaneously, 1 g of $Ca(OH)_2$ was added into 20 mL of ethanol and stirred at 50°C. After 2 h, 1 mL of distilled water was added, followed by 2 mL of TEOS. The silanization reaction was allowed to proceed for an additional 2 h at 70°C. Finally, the gelatin/chitosan and TEOS-functionalized $Ca(OH)_2$ solutions were mixed in a 1:1 ratio and freeze-dried for 24 h at −55°C. The final product was ground using a mortar and pestle. The physical appearance of the final composite was a white powder that was similar to that of unmodified $Ca(OH)_2$.

2.2. Material characterization

The gelatin/chitosan/TEOS/$Ca(OH)_2$ composite's chemical functional groups, crystallinity, morphology, and elemental mapping were studied using a Fourier-transform infrared (FTIR) spectrometer (Alpha II, Bruker, United States [US]), X-ray diffraction (XRD) system (Aeris, Malvern Panalytical, United Kingdom), and scanning electron microscopy (SEM) with energy-dispersive X-ray spectroscopy (SU3500, Hitachi High-Tech, Japan). For comparison, $Ca(OH)_2$ powder and purified $Ca(OH)_2$/TEOS compound were included in the analysis. $Ca(OH)_2$/TEOS compound was purified through centrifugation (3,000 rpm for 10 min) and washing in 10 mL of absolute ethanol at least 3 times, followed by drying in an oven at 40°C for 24 h.

2.3. Ca^{2+} release/dissolution

In the Ca^{2+} release experiment, 10 mg of either $Ca(OH)_2$ or gelatin/chitosan/TEOS/$Ca(OH)_2$ was immersed in 1.5 mL phosphate-buffered saline (PBS) at 37°C for 1, 5, 21, 93, and 196 h. At each observation time, the solution was refreshed by retrieving 1 mL of the solution and substituting it with an equal amount of fresh PBS. The 1 mL solution was diluted (10 times) using ultrapure water. The Ca^{2+} concentration in the diluted solution was measured using an atomic absorption spectroscopy (AAS; 3110, PerkinElmer Inc., USA) and inductively coupled plasma-optical emission spectrometry (ICP-OES; 5100, Agilent Technologies Inc., US). The Ca^{2+}

[1]Department of Pediatric Dentistry, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Yogyakarta, Indonesia; [2]Department of Dental Biomedical Sciences, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Yogyakarta, Indonesia; [3]Polytechnic Institute of Nuclear Technology, National Research and Innovation Agency, Sleman, Yogyakarta, Indonesia; [4]Research Centre for Food Technology and Processing, National Research and Innovation Agency, Gunung Kidul, Yogyakarta, Indonesia; [5]Research Centre for Polymer Technology, National Research and Innovation Agency, Tangerang Selatan, Banten, Indonesia; [6]Department of Oral Biology, Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Yogyakarta, Indonesia; [7]Faculty of Dentistry, Universitas Gadjah Mada, Sleman, Yogyakarta, Indonesia; [8]Department of Tissue Engineering and Regenerative Medicine, Faculty of Medicine, Universiti Kebangsaan Malaysia, Cheras, Wilayah Persekutuan Kuala Lumpur, Malaysia

concentration was determined using interpolation on a standard Ca^{2+} graph. Then, the Ca^{2+} release profile (per 10 mg sample) was calculated as a function of time according to Equation I:

$$Ca^{2+}\ (ppm) = C_i + C_{i-1} \quad (I)$$

where i = 1, 2, 3.,n, and C_i is the Ca^{2+} concentration at time i.

2.4. *In vivo* model of direct pulp capping

The study protocol involving an animal model was approved by the Ethics Committee of the Faculty of Dentistry and Prof. Soedomo Dental Hospital, Universitas Gadjah Mada (number: 58/UN1/KEP/FKG-RSGM/EC/2023). The approval was granted on May 09, 2023, and signed by the Committee Chairman, Dr. Ryna Dwi Yanuaryska. Animals were housed in individual cages under a controlled environment of the Animal Facility of the Integrated Experimental and Testing Laboratory at Universitas Gadjah Mada.

An exploratory animal study was designed to observe regenerative potential by comparing the expression of TGF-β1 scores and odontoblast-like cells across four different groups, including 7 days of $Ca(OH)_2$ or gelatin/chitosan/$Ca(OH)_2$/TEOS application, and 14 days of $Ca(OH)_2$ or gelatin/chitosan/$Ca(OH)_2$/TEOS application. A resource equation approach was applied to determine the minimum number of rats assigned to each group.[14] Based on the formula:

$$Minimum\ number\ of\ animal\ per\ group = \frac{10}{k} + 1 \quad (II)$$

Where k is the number of groups. The minimum number of animals per group for this study, after rounding, was four rats/group. Therefore, a total of 16 male Wistar rats (2–3 months old, 200–300 g) were proportionally assigned to four groups.

All animal treatment was performed under total sedation using ketamine hydrochloride 0.1 mL/100 g of body weight. Initially, the dental pulp of the first maxillary molar on the occlusal surface was intermittently drilled open using a diamond bur (No. 10, Edenta AG, Switzerland) mounted on a micromotor at a speed of 35,000 rpm without coolant. To stop bleeding, pressure was applied using a cotton pellet before placing the materials. Then, gelatin/chitosan/$Ca(OH)_2$/TEOS or $Ca(OH)_2$ was mixed with sterile water in a 1:1 (w/v) ratio. An applicator was used to place the material on top of the freshly opened pulp tissue, then covered with a temporary filling material.

2.5. Histological studies

After 7 and 14 days, the animals were sedated with a cocktail of ketamine hydrochloride 0.1 mL/100 g of body weight and xylazine hydrochloride 0.05 mL/100 g of body weight, and then euthanized. The molar teeth were harvested and kept in 10% buffered formalin fixative for 24 h. Then, the teeth were soaked in 10% ethylenediamine tetraacetic acid at pH 7.4 and 4°C for 4 weeks to allow decalcification. After softening, the tissues were trimmed vertically with a scalpel, removing connective tissues around the teeth, thereby obtaining approximately 0.5–1 cm of tissue. Subsequently, the tissues were rinsed under running water for 60 min. The next procedure involved making paraffin blocks and histological sections. Paraffin blocks were cut into 4 µm-thick sections with a microtome for immunohistochemical (IHC) staining, and 6 µm-thick sections for hematoxylin and eosin staining. The IHC staining kit, 2-step plus Poly-HRP Anti-Mouse/Rabbit IgG Detection System with DAB solution (E-IR-R217C, Elabscience Biotechnology, China), and the primary antibody for TGF-β1 (3C11 SC-130348, Santa Cruz Biotechnology Inc., US) were used according to the manufacturers' instructions.

A light microscope (YS100, Nikon, Japan) was used with the assistance of a camera (OptiLab SIGMA, PT Miconos, Indonesia) for histological evaluation. The TGF-β1 expression was observed at ×40 magnification and scored based on colour intensity, as shown in **Figure 1**. The scoring was incremented based on the intensity of brown colour in the histology slides, where score 0 represents no brown colour detected, score 1 represents light expression, score 2 represents medium expression, and score 3 represents the most vigorous intensity of colour. The quantification of odontoblast-like cells was conducted through observation at ×400 magnification, and the cells from three different fields of view were counted.

2.6. Data analysis

A 95% level of significance was employed for all statistical analyses in this study. The Shapiro–Wilk and Levene tests were applied before further analysis to test the normality and homogeneity of data. Given that the TGF-β1 data were not normally distributed and homogeneous, the Kruskal–Wallis and Mann–Whitney *post hoc* tests were applied to determine significant differences between the experimental groups. Alternatively, the analysis of variance (ANOVA) and the least significant difference test were used for odontoblast-like cell data that are normally and homogenously distributed.

3. Results

The gelatin/chitosan/$Ca(OH)_2$/TEOS was successfully synthesized and demonstrated controlled Ca^{2+} release ability.

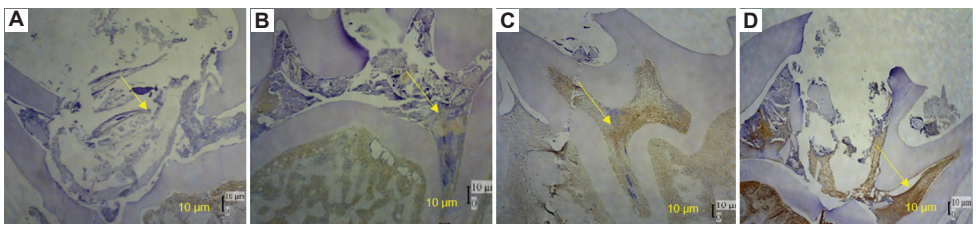

Figure 1. The guideline for colour intensity scoring of transforming growth factor β1 antibody expression. (A) Score 0 for no expression. (B) Score 1 for light expression. (C) Score 2 for medium expression. (D) Score 3 for strong expression. Scale bars: 10 µm; magnification: ×40.

The FTIR spectra (**Figure 2**) were collected to confirm the presence of functional groups in the materials at each synthesis step. The $Ca(OH)_2$ FTIR spectrum (black) showed the stretching mode of the O–H atoms, which was responsible for the narrow absorption at 3,645 cm^{-1}. Different vibration modes of C–O in carbonate groups CO_3^{2-} were ascribed to the absorption bands at 1,480, 1,080, and 873 cm^{-1}. Furthermore, the gaseous CO_2 was responsible for the small drop at 2,352 cm^{-1}. The presence of carbonate groups suggests that the sample was contaminated by atmospheric CO_2.[15] After functionalization with TEOS, the $Ca(OH)_2$/TEOS FTIR spectrum (red) was dominated by identical absorption bands in comparison to that of $Ca(OH)_2$. This phenomenon resulted from the former being mostly composed of the same $Ca(OH)_2$, with TEOS functionalization only on the surface. On closer examination of individual absorption bands, however, a small drop was discernible at 1,080 cm^{-1}, which corresponds to the characteristic Si–O–Si band of the silica network.[16] The spectrum of gelatin/chitosan/$Ca(OH)_2$/TEOS composite (blue) comprises stretching vibration of O–H and/or N–H at 3,400 cm^{-1}, C=O of primary amide at 1,611 cm^{-1}, and C–N at 1,550 cm^{-1},[17] typical of both gelatin and chitosan. Importantly, the Si–O–Si band of the silica network was even stronger at 1,080 cm^{-1}.

The SEM imaging demonstrated the morphology of $Ca(OH)_2$, $Ca(OH)_2$/TEOS, and gelatin/chitosan/$Ca(OH)_2$/TEOS composite, as seen in **Figure 3**. In general, no distinct morphology was observed between $Ca(OH)_2$ (**Figure 3A** and **D**) and $Ca(OH)_2$/TEOS (**Figure 3B** and **E**). As previously mentioned, this phenomenon results from the former being predominantly composed of the same $Ca(OH)_2$, with TEOS functionalization only on the surface, most probably at a nanoscale. Distinct morphology was observed after incorporation of the $Ca(OH)_2$/TEOS into the gelatin/chitosan polymer matrix (**Figure 3C** and **F**) compared with the other panels.

Elemental mapping was performed to validate the presence of key elements in each of the studied materials. It is seen in

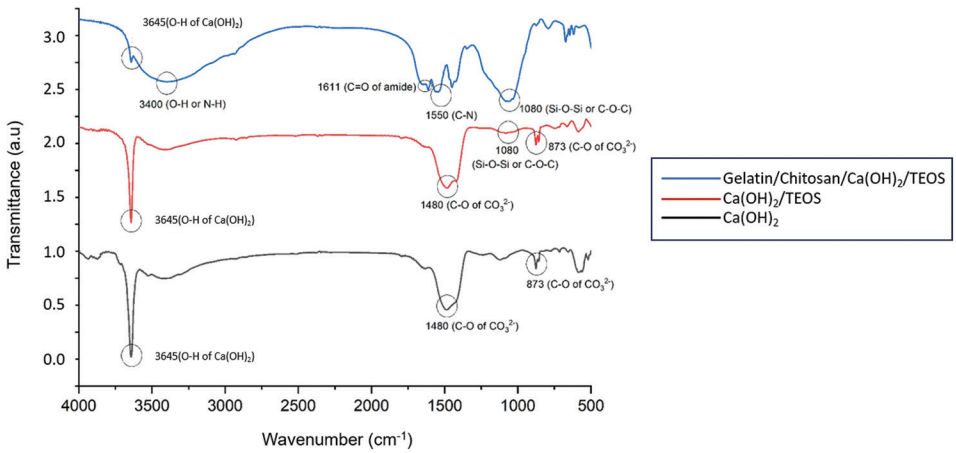

Figure 2. Fourier transform infrared spectra of $Ca(OH)_2$ (black), $Ca(OH)_2$/TEOS (red), and gelatin/chitosan/$Ca(OH)_2$/TEOS composite (blue). Circles indicate O–H band at 3,400 and 3,645 cm^{-1}, C=O at 1,611 cm^{-1}, C–N at 1,550cm^{-1}, C–O at 1,480 and 873 cm^{-1}, and Si–O–Si or C–O–C at 1,080 cm^{-1}.
Abbreviation: TEOS: Tetraethyl orthosilicate.

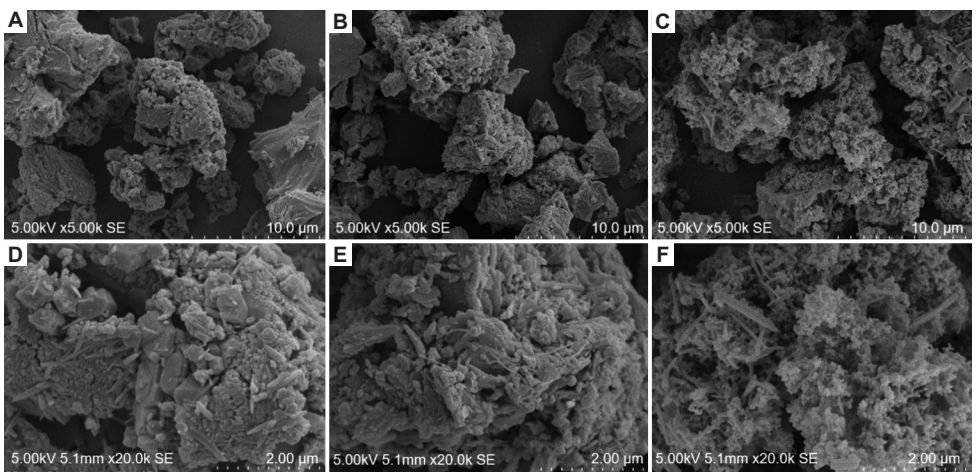

Figure 3. Scanning electron microscopy images of (A and D) $Ca(OH)_2$, (B and E) $Ca(OH)_2$/TEOS, and (C and F) gelatin/chitosan/$Ca(OH)_2$/TEOS composite. No structure difference between $Ca(OH)_2$ and $Ca(OH)_2$/TEOS. In contrast, the gelatin/chitosan/$Ca(OH)_2$/TEOS composite forms bigger bulks. Scale bar: (A-C) 10.0 µm, (D-F) 2.00 µm; magnification: (A-C) ×5,000, (D-F) ×20,000.
Abbreviation: TEOS: Tetraethyl orthosilicate.

Figure 4A that $Ca(OH)_2$ was primarily composed of Ca (66.7%) and O (33.3%) elements, as expected. However, a minor amount of C and N was observed. After careful investigation, it was concluded that the detected C and N elements originated from the double-sided tape used to support the $Ca(OH)_2$ powder during the acquisition. Due to this reason, the C and N atomic percentage data were omitted from the calculation.

Functionalization of the $Ca(OH)_2$ surface with TEOS resulted in the occurrence of Si element, which is expected, and an increased value of C (14.6%), most probably originating from the unhydrolyzed organic ligand residue of TEOS (**Figure 4B**). Other elements and their atomic percentages were O (66.7%), Si (0.2%), and Ca (18.5%). After incorporation of the $Ca(OH)_2$/TEOS into the polymer matrix, the atomic percentage of C (25.7%) and O (43.4%) increased significantly, and the occurrence of N (20.6%) was observed due to the gelatin/chitosan molecules (**Figure 4C**). In addition, Si (3.2%) and Ca (7.1%) elements were observed. These results elucidate the chemical structure of the gelatin/chitosan/$Ca(OH)_2$/TEOS composite.

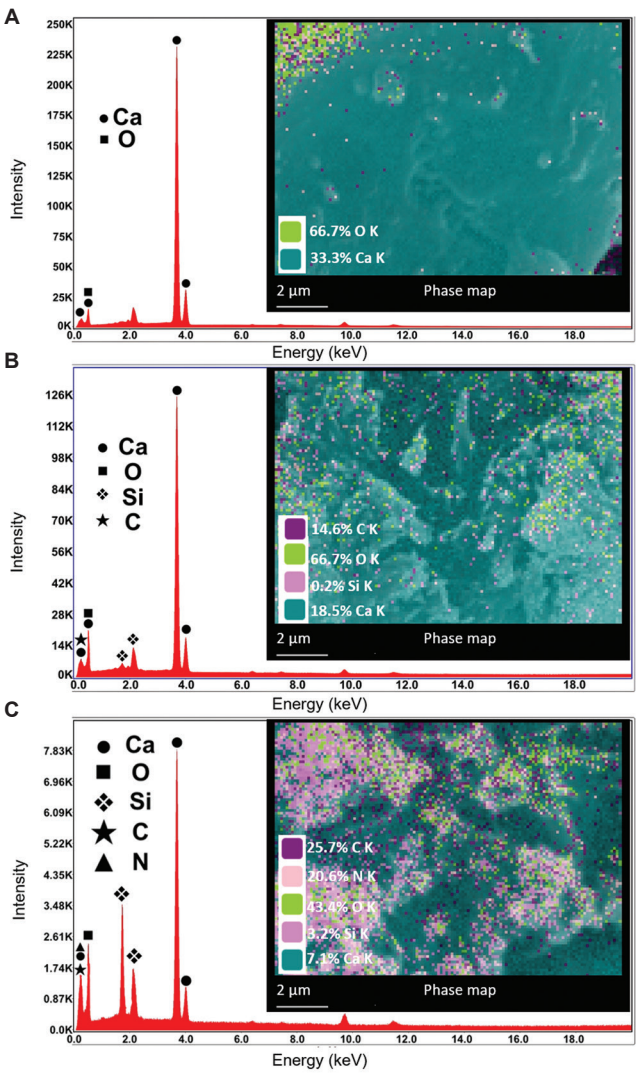

Figure 4. Elemental mapping and atomic percentage of (A) $Ca(OH)_2$, (B) $Ca(OH)_2$/TEOS, and (C) gelatin/chitosan/$Ca(OH)_2$/TEOS composite. Si and O elements indicate the presence of TEOS in $Ca(OH)_2$/TEOS and gelatin/chitosan/$Ca(OH)_2$/TEOS. Additionally, C and N elements are identified in the result of gelatin/chitosan/$Ca(OH)_2$/TEOS composite, as gelatin and chitosan were incorporated.
Abbreviation: TEOS: Tetraethyl orthosilicate.

The XRD pattern of $Ca(OH)_2$ (**Figure 5**, black) revealed all the characteristic peaks of $Ca(OH)_2$ with the 2θ values of 18.11° (0 0 1), 28.72° (1 0 0), 34.11° (1 0 1), 47.17° (1 0 2), 50.82° (1 1 0), 54.38° (1 1 1), 62.63° (2 0 1), and 64.32° (1 1 2). These characteristic peaks were compared to the standard ICDD database (#00-004-0733).[18,19] After incorporating the TEOS-functionalized $Ca(OH)_2$ into the polymer matrix, the composite became highly amorphous; however, with typical $Ca(OH)_2$ crystalline peaks still observed. In addition, a small peak, typical of a silica network synthesized from TEOS, was observed at 2θ of 21.76°.[20]

The composite in this study was designed to reduce $Ca(OH)_2$ solubility and control the amount of Ca^{2+} released into the environment. The $Ca(OH)_2$ particles were functionalized with TEOS, potentially creating a Si–O–Si network on their surface and connecting them covalently with the polymer matrix. This strategy was expected to sustain the release of Ca^{2+} in comparison to $Ca(OH)_2$ particles without either crosslinker or matrix. In the Ca^{2+} release experiments, a 10 mg sample was immersed in 1.5 mL PBS, and the concentration of Ca^{2+} in the solution was measured from time to time.

Figure 6 presents the Ca^{2+} release profile of the $Ca(OH)_2$ group. In contrast, Ca^{2}□ release from the gelatin/chitosan/$Ca(OH)_2$/TEOS composite was not detected, as the concentration remained below the detection limits of both AAS (approximately 0.05 mg/L) and ICP-OES (approximately 0.01 mg/L). This suggests an extremely low solubility of Ca^{2+} in the composite system, potentially due to physicochemical entrapment and reduced loading. A longer incubation period or the use of more sensitive analytical techniques may be required to detect the delayed or localized release. In addition, the release profile of the $Ca(OH)_2$/TEOS intermediate group was not included, as the TEOS modification forms only an

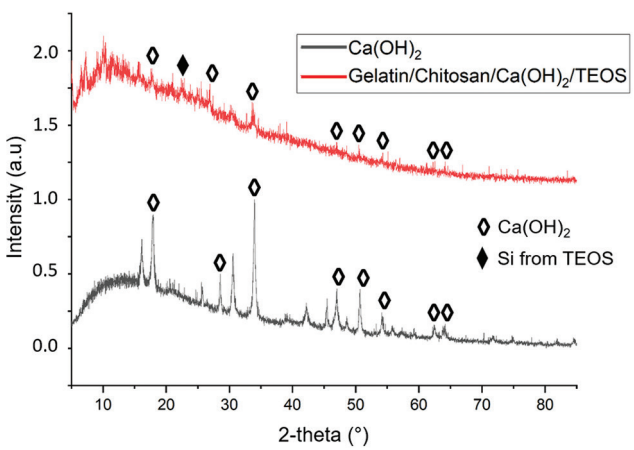

Figure 5. X-ray diffraction diffractograms of $Ca(OH)_2$ (black) and gelatin/chitosan/$Ca(OH)_2$/TEOS composite (red) show the structural differences between the two materials
Abbreviation: TEOS: Tetraethyl orthosilicate.

ultrathin surface layer that does not significantly alter the bulk release behaviour compared to unmodified $Ca(OH)_2$.

Seven days after material application on the open dental pulp model, TGF-β1 was expressed at a low intensity in the $Ca(OH)_2$ group but at a strong intensity in the composite group, acquiring Score 1 and Score 3, respectively. The result persisted for another 7 days, that is, 14 days after the material was applied. In **Figure 7**, the most intensive expression from each group was demonstrated. A significant difference was found between the experimental groups at both 7 days (p=0.022) and 14 days (p=0.022) of evaluation. In contrast, when comparing the results between day 7 and day 14 from the same group, the TGF-β1 expression scores for both $Ca(OH)_2$ (p=1.000) and the composite (p=1.000) groups were not significantly different.

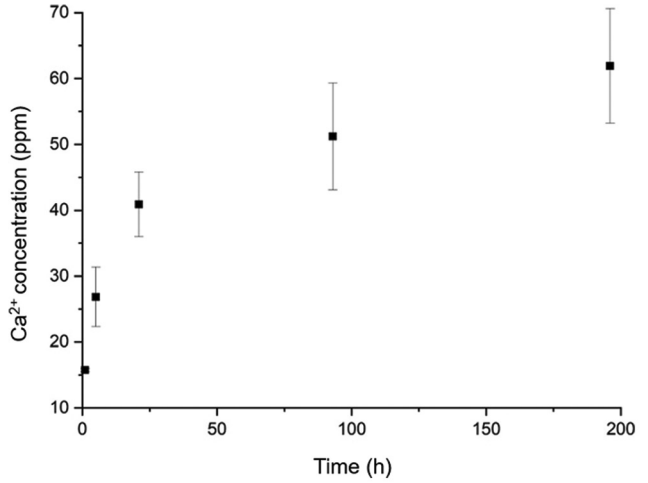

Figure 6. Ca^{2+} release profile of 10 mg $Ca(OH)_2$ in 1.5 mL phosphate-buffered saline over time. Ca^{2+} release increases over time.

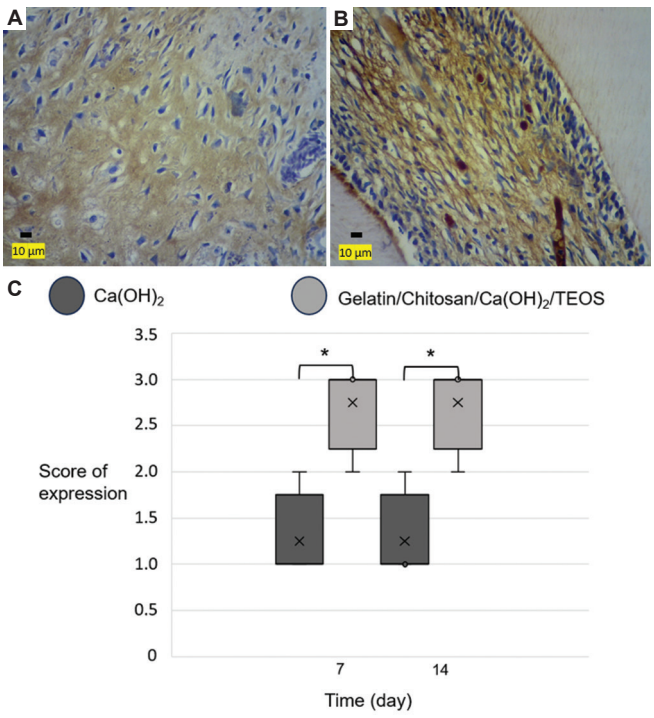

Figure 7. Expression of transforming growth factor-β1 in dental pulp tissues. (A) $Ca(OH)_2$ group. (B) Gelatin/chitosan/$Ca(OH)_2$/TEOS composite group. Scale bars: (A and B) 10 μm; magnification: (A and B) ×400. (C) The comparison of scoring results between groups after 7 and 14 days of application.
Note: *p<0.05.
Abbreviation: TEOS: Tetraethyl orthosilicate.

Data of odontoblast-like cell quantification were normally distributed (p=0.934) and homogenous (p=0.538). The average number of odontoblast-like cells of both $Ca(OH)_2$ and composite groups showed an increasing trend from day 7 to day 14 after material application (**Figure 8**). However, the increment was only statistically significant in the $Ca(OH)_2$ group (p<0.001). When compared at each evaluation time, the odontoblast-like cell number of the $Ca(OH)_2$ group was significantly lower than that of the composite group (p=0.020) at day 7 but not at day 14 (p=0.100). The ANOVA results showed that neither $Ca(OH)_2$ nor the composite led to a significantly different number of odontoblast-like cells (p=0.540), but the duration of application increased it significantly (p=0.002). The analyses also revealed an interaction between material types and duration on the number of odontoblast-like cells.

4. Discussion

A delivery system of $Ca(OH)_2$ was successfully synthesized using gelatin and chitosan as the matrix and TEOS to bind the inorganic compound to the organic backbone. The FTIR spectra and SEM/EDX mapping showed that the final composite was composed of the expected chemical compounds that were recognized as gelatin, chitosan, and $Ca(OH)_2$. The Si–O–Si band demonstrated TEOS's roles as a crosslinker through silica network development. The morphology of $Ca(OH)_2$ from the SEM images agrees with that in the literature.[21] The composite exhibited a different physical structure. This was confirmed using the XRD diffractogram, which identified that the composite was highly amorphous and had lower crystallinity compared to $Ca(OH)_2$.

The exposure of dental pulp triggers inflammatory cascades and nerve degeneration.[4,10,11] When applied to the animal model, both materials were in direct contact with a freshly opened dental pulp. The strategy of developing a delivery system using gelatin/chitosan has been shown to induce milder responses compared to those induced by $Ca(OH)_2$. The previous study by Abdulrahman *et al.*[11] indicated that the expression of pro-inflammatory markers, cyclooxygenase-2 and tumor necrosis factor-α, was reduced in the gelatin/chitosan/$Ca(OH)_2$/TEOS-treated groups. The interaction between the composite and the exposed pulp tissue also triggered less expression of nerve degeneration markers, namely, nestin[10] and protein gene product 9.5.[11] These findings demonstrate the ability of the composite to protect against excessive inflammation response after an acute injury.

In this study, injury was promoted by drilling the enamel and dentin layers to expose the dental pulp. The native odontoblast layer, located right underneath the dentin, was severely damaged. Theoretically, the capacity of regeneration was influenced by the intensity of injury and the involvement of microbes. Applying material directly on an open pulp without

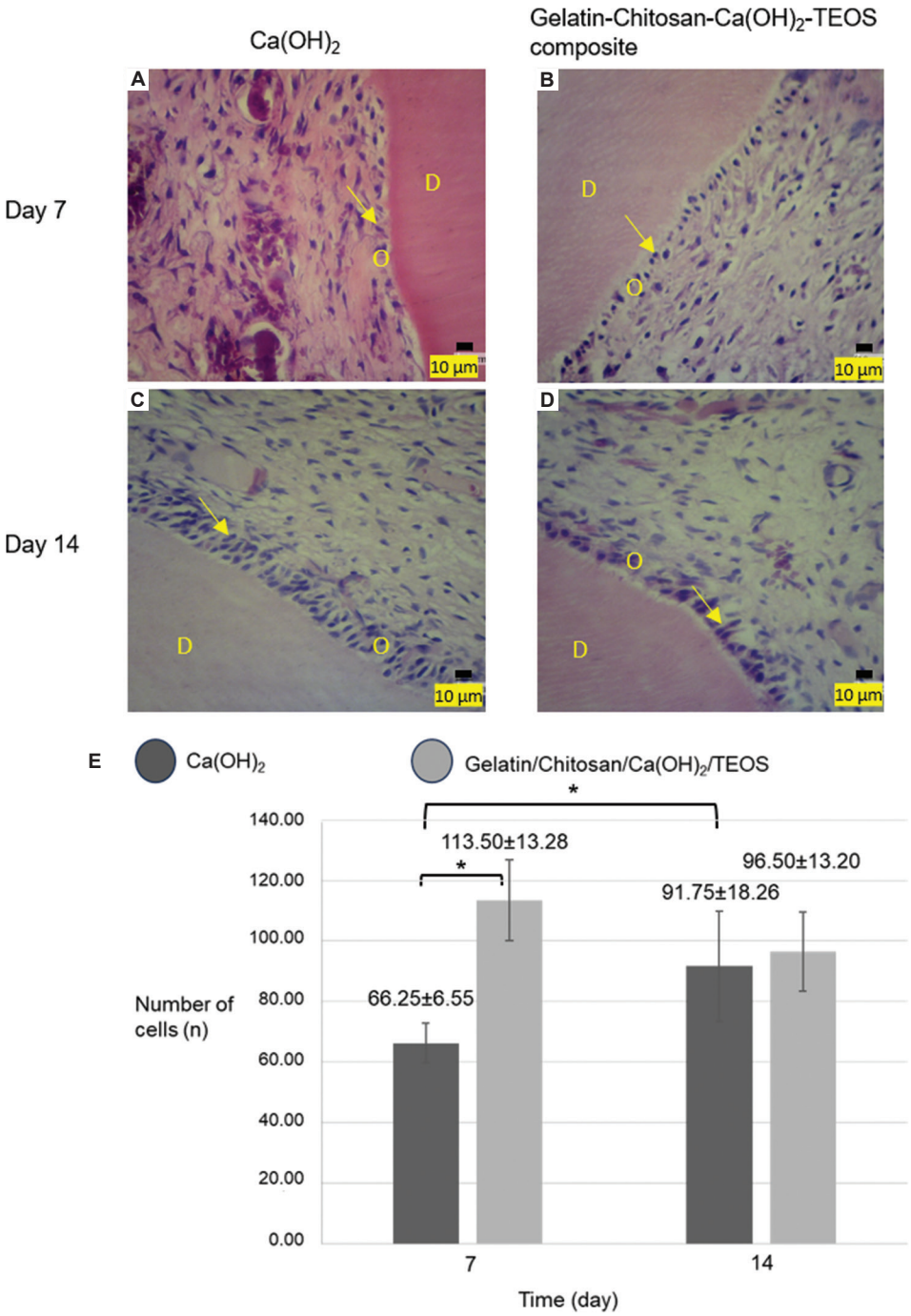

Figure 8. Dental pulp tissues after 7 and 14 days of $Ca(OH)_2$ or gelatin/chitosan/$Ca(OH)_2$/TEOS composite application. (A-D) Hematoxylin and eosin staining under ×400 magnification of a light microscope (scale bar: 10 µm). D indicates dentin, O indicates the odontoblast layer, and yellow arrows indicate the odontoblast-like cells. (E) Quantification of odontoblast-like cells.
Note: *p<0.05.
Abbreviation: TEOS: Tetraethyl orthosilicate.

meticulously removing the infected dentin may lead to pulp necrosis.[3] However, this study considered that the infected dentin was minimal, as the pulp opening was not induced by a caries and the material was applied immediately after injury. Nonetheless, microbial contamination from saliva remained a potential risk factor. Considering this perspective, the antimicrobial effect of chitosan and $Ca(OH)_2$ was widely recognized. Chitosan has been shown to possess the capacity to inhibit oral microorganisms, such as *Streptococcus mutans*, *Streptococcus sobrinus*, *Streptococcus sanguis*,[22] *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Candida albicans*.[23] On the other hand, it has recently been reported that $Ca(OH)_2$ inhibited the growth and biofilm formation of clinically isolated *S. mutans*, *S. sobrinus*, and *Enterococcus faecalis*.[24] A study comparing the antimicrobial capacity of the composite and unmodified $Ca(OH)_2$ was recommended, specifically against caries-related bacteria, namely, *S. mutans*, *S. sobrinus*, *S. sanguis*, and dental root canal bacteria *E. faecalis*.

Human DPSCs differentiate into odontoblast-like cells, initiating the sequence of the regeneration process after injury.[3-5] An *in vitro* study on primary human DPSC culture demonstrated that $Ca(OH)_2$ significantly promoted cell proliferation and enhanced human DPSC differentiation into odontoblast-like cell lineage after 72 h.[25] Our *in vivo* study displayed that the number of odontoblast-like cells increased significantly 14 days after $Ca(OH)_2$ application. Interestingly, although the Si–O–Si network covalently bonded $Ca(OH)_2$ to the gelatin/chitosan matrix and evidently reduced its solubility, the number of odontoblast-like cells in the composite group still significantly increased. When comparing both materials, the composite promoted a significant elevation on days 7 and 14. Hypothetically, gelatin and chitosan contributed to the differentiation, and environmental Ca^{2+} was not the most crucial factor. Moreover, the delayed Ca^{2+} may be more advantageous in the late phase of dental pulp regeneration, specifically in the mineralization of hard tissues.

Although Ca^{2+} release from the composite was consistently undetectable in the bulk medium, the regenerative histological responses observed *in vivo* suggest localized bioavailability of calcium at the dentin–pulp interface. This may reflect microenvironmental release dynamics not captured by the detection methods used in this study. In addition, the relatively low total $Ca(OH)_2$ content in the composite (due to dilution by gelatin, chitosan, and TEOS) further explains the suppressed solubility. These findings support the hypothesis that bioactivity was mediated by both the localized calcium signaling and the inherent biological properties of the matrix components.

The DPSCs' transformation into odontoblast-like cells was a result of TGF-β1 stimulation on protein kinase B, extracellular signal-regulated kinase1/2, and p38 mitogen-activated protein kinase signaling pathways. Consequently, this increased the expression of dentin matrix protein-1, collagen type 1, and alkaline phosphatase.[13] The TGF-β1 expression was not significantly elevated after 7 days, compared to that after 14 days, in both experimental groups. However, when comparing the expression on each experimental day, the composite induced significantly stronger expression. This again showed the regenerative effect of the delivery system.

5. Conclusions

It can be concluded that the gelatin/chitosan.$Ca(OH)_2$/TEOS composite shows potential in regenerating dental pulp tissues after injury by increasing TGF-β1, which is related to the differentiation of DPSCs into odontoblast-like cells. However, parameters assessed in this study reflect only the early stage of dental pulp regeneration and are insufficient to demonstrate the benefit of a delivery system in achieving natural-like tissue formation. Future long-term cohort studies are imperative for investigating regenerative outcomes, including apical closure, dentin reparative formation, and tooth vitality, parallel with a histology study to ensure regeneration at the microstructural level. In addition, the antimicrobial property of both materials is interesting to compare.

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The authors declare no conflicts of interest.
Author contributions
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Ethics approval and consent to participate
The study protocol involving an animal model was approved by the Ethics Committee of the Faculty of Dentistry and Prof. Soedomo Dental Hospital, Universitas Gadjah Mada (number: 58/UN1/KEP/FKG-RSGM/EC/2023).
Consent for publication
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Availability of data
Data are available from the corresponding author on reasonable request, and animal experiment data are provided as supplementary information.

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