Biological effects of α-TCP/Te-CP cement as a pulp-capping agent

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ORIGINAL ARTICLES

Biological effects of α-TCP/Te-CP cement as a pulp-capping agent

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In this study, we investigated the affinity of our calcium phosphate cements to dental pulp cells. The cement was mainly composed of α-TCP and Te-CP, prepared by a one-step sintering method and mixed with three different aqueous solutions. Three types of α-TCP/Te-CP cements, each with 1 M or 2 M sodium dihydrogen phosphate, or 1 M citric acid, and calcium hydroxide agent were evaluated.

First, dental pulp cells were cultured in a medium containing components leached from the cements, and we found that our all three types of α-TCP /Te-CP cement did not inhibit cell proliferation and showed no significant cytotoxicity compared to the calcium hydroxide cement. In vivo evaluation was then performed using a pulp-exposed animal model. The α-TCP /Te-CP cement prepared with 2M sodium dihydrogen phosphate showed remarkable pulpal tissue affinity. These results suggested that the newly developed α-TCP /Te-CP material could be a promising pulp capping material.

Key words : one-step sintering, α-TCP/Te-CP cement calcium phosphate, dental pulp capping, dental pulp stem cell

1 Introduction

Preservation of the dental pulp is important for the long-term preservation of teeth. When the pulp is exposed due to caries, it is difficult to preserve even a small amount, ultimately requiring pulp removal. However, if accidental exposure occurs during cavity preparation or accompanying tooth fracture, and if the diameter of the exposed area is 2 mm or less and only a short period of time has passed after the initial exposure, it may be possible to use direct pulp capping to conserve the pulp, thereby making the recovery and maintenance of a healthy state possible[1)]. In such cases, calcium hydroxide is most commonly used as a direct pulp-capping agent[1-4)]. Although this preparation is considered to have marked antibacterial properties[4)], which enable the rapid formation of a hard-covering tissue and promote healing and closure of the exposed area[3, 5)], its strong alkaline properties result in significant irritation of the dental pulp and may cause extensive pulp necrosis[6-8)]. On the other hand, in recent years, trioxide inorganic aggregate (MTA) cements have been widely used and highly regarded in the field of endodontics[9, 10)]. A statistical study has reported that MTA cements have a higher success rate and less inflammatory response than calcium hydroxide cements[11)]. This report also noted that dental adhesive systems showed lower success rates

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for MTA compared to calcium hydroxide cement. The efficacy of alkaline stimulation of calcium hydroxide cement is also debatable. Thus, the most suitable pulp capping material has not been established. Therefore, many researches are underway to develop new pulp capping agents that are biocompatible and are expected to promote the dentin bridge formations[12-16]. Because of their similarity to the hydroxyapatite (HA) mineral phase of natural bones and teeth, dental cements containing calcium phosphate are expected to promote dentin remineralization and have been studied extensively in recent years[14, 15, 17-19]. Various types of calcium phosphates have been reported to exhibit high biocompatibility [15, 17]. In addition, many studies have shown that multi-component calcium phosphate cements stimulate the dental pulp and induced the formation of reparative dentin[14, 17, 19, 20]. Calcium phosphate cements of various compositions, not only those consisting of a single calcium phosphate compound, have been developed for dental and medical applications. The different compositions of multi-component cements indicate their different properties[18-20].

Recently, a number of cements with two or more calcium and phosphoric acid compounds as the solid phase have become commercially available. In order to prepare these cements with multi-components different calcium phosphate compounds should be mixed mechanically after each calcium phosphate was synthesized. To avoid this problem, we reported a one-step sintering method for preparing cement powders of multi-component calcium phosphate mixtures[21]. In addition, we reported that the α-tricalcium phosphate and tetracalcium phosphate (α-TCP /Te-CP) mixtures, the molar ratio of two calcium phosphate compounds, equivalent to 1/1, 1/2, and 1/8, could be synthesized in one step method, in which heating was performed only one time[22]. Eight types of α-TCP/Te-CP (molar ratio 1/2) cements obtained in the study above mentioned were prepared with aqueous solutions containing sodium dihydrogen phosphate, citric acid, or orthophosphoric acid, including those with different powder-liquid ratios, and their effects as a pulp capping agent was evaluated[23]. Results of in vivo assay, at 7 days after the pulp lining, cement with hydrogen phosphate solution as mixing solution, and slightly later, at 14 days, cement with citric acid solution as mixing solution, showed dentin bridge formation comparable to that of calcium hydroxide agent[23]. However, the evaluation of pulp cell and tissue responses is still lacking, and the effects of these cements with different admixtures remain unclear.

In this study, we prepared three types of α-TCP/Te-CP cements with different admixture solutions and investigated the cell biological effects of these cements and calcium hydroxide agent on the human dental pulp stem cells (hDPSCs). In addition, the response of pulp tissue was also examined histochemically in an animal model, and the cement mixture was evaluated for the use of α-TCP/Te-CP cement as a pulp capping agent.

2 Materials and Methods

2. 1 Preparation of the α-TCP/Te-CP cements

Calcium carbonate ($CaCO_3$) and dicalcium phosphate dihydrate ($CaHPO_4{\cdot}2H_2O$) were mechanically mixed in a molar ratio of 5: 6 to ensure that the post-firing molar ratio of 1:2 for α-TCP to Te-CP[22]. After mixing, the materials were heated for 5 h at 1500 ℃ . These processes were performed under the same conditions based on our previous study[22] that identified the product after firing as a mixture of α-TCP and Te-CP. The fired block was then crushed and graded, and particles of 74 μm or smaller were used as cement powder. We prepared a 1 M sodium dihydrogen phosphate aqueous solution, 2 M sodium dihydrogen phosphate solution, and 1 M citric acid solution as cement mixing solutions. Cement A was prepared by mixing the 1 M sodium dihydrogen phosphate with cement powder at a powder-to-liquid ratio (P/L) of 2.0. Cement B was prepared by mixing the 2 M sodium dihydrogen phosphate with cement powder at a P/L ratio of 1.5. Cement C was prepared by mixing the 1 M citric acid with cement powder at a P/L ratio of 2.5. For the control group, we used a commercially-available calcium hydroxide pulp capping agent (Dycal®, DENTSPLY, New York, NY, USA).

2. 2 Preparation of experimental cell culture medium

Cylindrical acrylic rings with an internal diameter of 10 mm, an external diameter of 16 mm, and a height of 5 mm were prepared. After mixing, the prepared cements were used to fill the acrylic rings, which were then soaked for 24 h in 5.0 ml of phenol red free α-modified minimal essential medium (α-MEM) at

37 ℃. The supernatant was then sterilized through a 0.22-μm MILLEX® GV Filter Unit (Merck Millipore, Darmstadt, Germany). After sterilization, a small amount of the prepared medium was used to measure the concentration of Ca and P. The QuantiChrom™ Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA) was used to measure the concentration of Ca, and the Phosphate Assay Kit (BioChain, Newark, CA, USA) was used to measure the concentration of P, according to the instructions. From the remaining medium, 4.05 ml was measured and 0.45 ml of fetal bovine serum (FBS) was added to obtain 4.5 ml of α-MEM containing 10% FBS, which was used for the cell culture experiments. Serial dilutions (1/2, 1/4, up to 1/64) of this medium were then made in α-MEM. Both kits for measuring Ca and P concentrations are based on the colorimetric method. The absorbance of each was measured with a plate reader according to the instructions, and the concentrations of both in the experimental medium were calculated from a standard curve prepared using the measurements of the standard solutions provided in the kits.

2. 3 Evaluation of the hDPSC activities

The hDPSCs (Allcells, Emeryville, CA, USA), which had been subcultured in α-MEM containing 10% FBS, were seeded at 4,000 cells/well in a 96-well plate. Six hours after seeding, the medium was replaced to experimental medium containing graded concentrations of cement eluate. After further incubation for 24 h, a WST-1 reagent (Cell Counting Kit, Dojindo Laboratories, Kumamoto, Japan) was added to the medium, and a spectrophotometer (Infinite Pro-200, TECAN, Tokyo, Japan) was used to measure the absorbance at 450 nm; subsequently, the hDPSC growth index was determined. The absorbance of cell-free MEM and four undiluted culture mediums containing cement lysate were used as background controls (blank), and the absorbance of the medium containing cell lysate after the WST-1 reagent reaction was measured. The value of the blank control was subtracted from the measured value as the data. The hDPSCs were cultured using the same method as that used for growth evaluation. We investigated the activity of lactate dehydrogenase (LDH), which leaks out of living cells when the cell membrane is damaged. We used a cytotoxicity detection kit (Takara Bio Inc., Otsu, Japan) and measured the absorbance at 492 nm to evaluate the cytotoxicity.

2. 4 Calcein and propidium iodide staining

To evaluate the survival of cells in the experimental medium, the hDPSCs were cultured in the same manner as above in 3.5-cm glass-bottom dishes (Greiner Japan, Tokyo, Japan). After incubation of the cells for 24 h, calcein, which is specifically used to stain living cells, and propidium iodide (PI), which is used to stain dead cells, were added to the culture with a final concentration of 1 μg/ml. After 30 min, the cells were observed under a Ti U microscope (Nikon, Kawasaki, Japan).

2. 5 Observation of the cell morphology

To observe the effects of the experimental medium on cell morphology, the hDPSCs were cultured in the same manner as described above. After 24 h of culture, cells were fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and subjected to membrane permeation treatment for 15 min in PBS containing 0.1% Triton X-100. To detect vinculin[24], a protein that forms focal desmosomes, we used an anti-human vinculin mouse monoclonal antibody (Sigma, St. Louis, MO, USA) diluted to 1/100 as the primary antibody and an Alexa 594-labeled anti-mouse IgG antibody (Life Technologies Japan, Tokyo, Japan) diluted to 1/1000 as the secondary antibody. Immunofluorescence staining and actin filament staining were performed using Alexa 488-labeled phalloidin (Life Technologies), as previously reported[25]. The nuclei were then stained using DAPI (Dojindo Laboratories). The cells were subsequently observed under a confocal laser-scanning microscope (LSM-710, Carl Zeiss, Jena, Germany). We also randomly selected cells and measured the pericellular length and area based on the results of actin filament staining using the Lumina Vision analysis software (Mitani Corporation, Tokyo, Japan).

2. 6 Histochemical evaluation of the exposed pulp model

Artificial exposure sites were prepared in the right upper first molars of 20 Wistar rats, 8-week-old male (SLC, Hamamatsu, Japan) according to a previous report[28]. The process is briefly described as follows: Rats were anesthetized by inhalation

of isoflurane followed by general anesthesia with intraperitoneal administration of a triad of anesthetics (1.0 mg medetomidine hydrochloride (Dormitor, Meiji Animalhealth, Kumamoto, Japan), 0.375 mg/kg midazolam (Sandz, Tokyo, Japan), 2 mg/kg butorphanol tartrate (Vertofar, Meiji Animalhealth). The occlusal surface of the maxillary right first molar was punctured by 0.9 mm in diameter sterile diamond round-point bar attached to a Surgical motor Pro (Straumann Japan, Tokyo, Japan), followed by rinsing with 6% sodium hypochlorite and 3% hydrogen peroxide solution and then with saline solution. After the affected area was hemostatic, direct pulp capping was performed using the four types of cement, and after 7 days, the maxillary right first molar and surrounding tissues were extracted and paraffin-embedded sections were prepared. According to routine methods, the sections were deparaffinized and subjected to hydrophilic treatment. A TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed to detect fragmented DNA as a marker of cell death. The DeadEndTM colorimetric apoptosis detection system (Promega, Madison, WI, USA) was used for staining. For the histochemical evaluation of proliferating cells, proliferation cell nuclear antigens (PCNAs) (DAKO Japan, Tokyo, Japan) were used for immunostaining in accordance with a previous report [26]. All animal experiments were approved by the Ethics Committee for Animal Experiments of Asahi University (approval number, 20-043).

2.7 Statistical analysis

Statistical analysis was performed using the KaleidaGraph software (ver. 4.0, Hulinks Inc., Tokyo, Japan). All data shown are presented as the mean ± standard deviation. All experiments were performed at least twice. Analysis of variance was used to investigate the statistical significance of the data. Subsequently, Scheffé's multiple comparison test was performed, and a p-value < 0.05 was considered statistically significant.

3 Results

3.1 Evaluation of cell growth in the experimental medium

The Ca and P concentrations of the control α-MEM and the experimental medium after cement immersion are shown in Table 1. All four experimental media showed higher calcium concentrations than the control α-MEM, confirming calcium leached into the media. Among them, Cement D experimental medium had the highest calcium concentration. On the other hand, the phosphorus concentration in the Cement D experimental medium was not different from that of the control α-MEM, whereas the calcium phosphate cement, A-C experimental media showed leaching of phosphorus. Among them, Cement B experimental medium had the highest phosphorus concentration. In Cement A and B experimental medium, cell growth was not significantly different from the cells cultured in α-MEM, as a negative control (NC), regardless of cement eluate concentration (Figure 1A and B). In the Cement C experimental medium, although, slight growth inhibition was observed in the medium with the highest concentration of cement eluate, but no statistically significant difference was observed between the other media and the NC media. (Figure 1C). Cell growth was significantly inhibited in the experimental medium containing eluate from calcium hydroxide cement to NC (Figure 1D).

3.2 Cytotoxicity of eluates from the experimental cement

No significant differences in LDH activity were noted between the NC and any cell culture with the Cement A, B, and C experimental medium (Figure 2A-C). However, in the medium containing eluates from calcium hydroxide cement, LDH leakage

Table 1 The concentration of Ca and P of α-medium and cement-immersed experimental media

Sample Label	α-medium	A	B	C	D
Ca (mM)	1.8 ± 0.08	2.2 ± 0.19	2.1 ± 0.14	2.2 ± 0.4	2.3 ± 0.23
P (mM)	1.0 ± 0.03	1.4 ± 0.26	1.7 ± 0.22	1.0 ± 0.03	1.0 ± 0.04

Data are means of three times measurements of each sample.

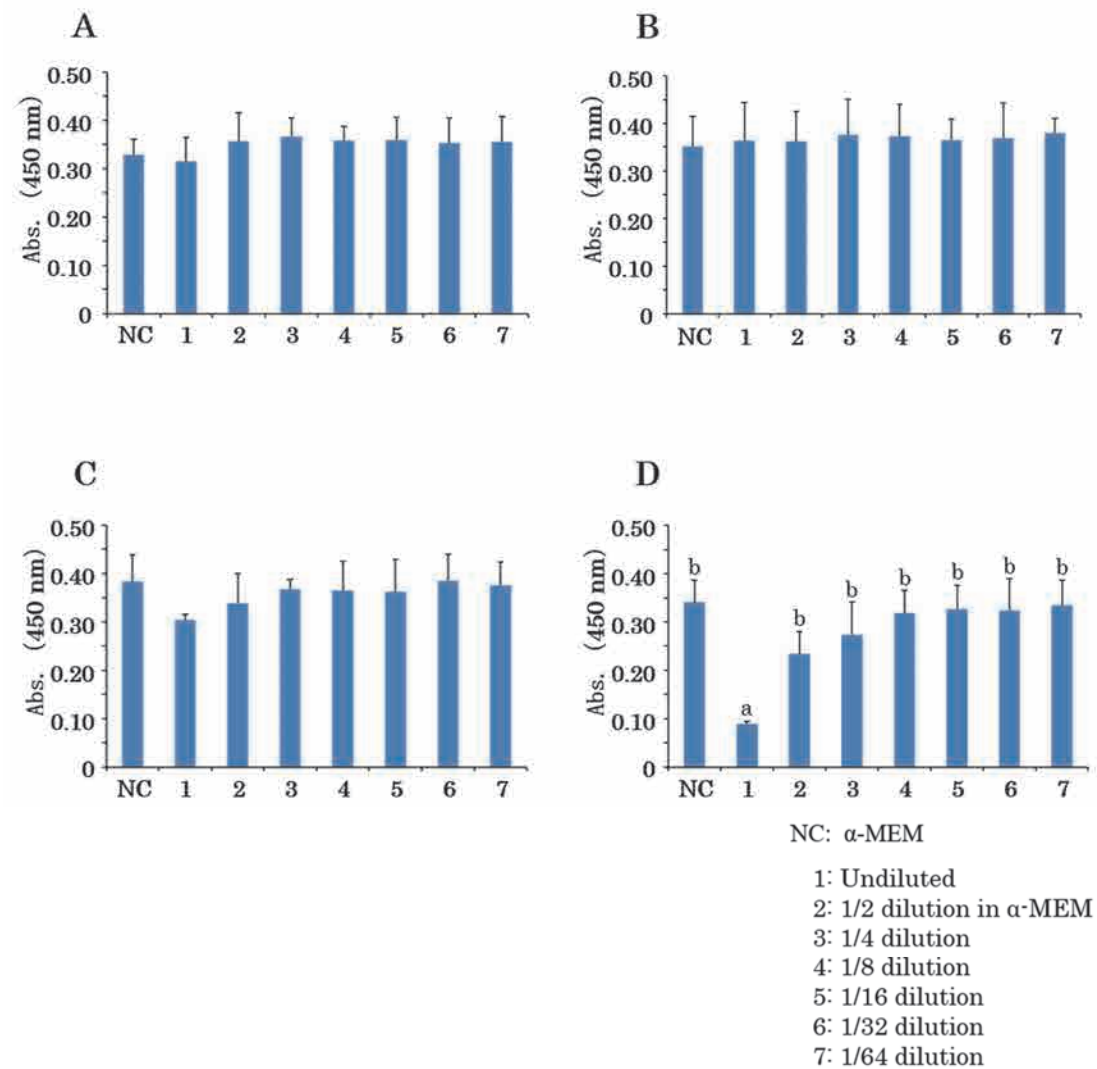

Figure 1　Evaluation of the growth of hDPSCs in the experimental medium

A: Growth of hDPSCs in the Cement A experimental medium.

B: Growth of hDPSCs in the Cement B experimental medium.

C: Growth of hDPSCs in the Cement C experimental medium.

D: Growth of hDPSCs in the calcium hydroxide experimental medium.

The graph shows the mean values and standard deviations for each group (n = 4).

Statistically significant differences are indicated by different letters ($p < 0.05$).

significantly increased at concentrations above a certain level (sample label, 1–4) (Figure 2D).

3.3 Live and dead evaluation of hDPSCs in the experimental culture media

The hDPSCs cultured in the NC medium were calcein positive and fusiform. No dead propidium iodide-positive cells were detected. While the hDPSCs cultured in Cement A experimental culture medium exhibited some weak PI-positive cells, hardly any PI-positive cells were observed among the hDPSCs cultured in the Cement B experimental medium, and the live cells exhibiting calcein positivity had a fusiform shape (Figure 3). Additionally, many PI-positive cells were observed in Cement C and the calcium hydroxide media. In particular, among the hDPSCs cultured in the calcium hydroxide medium, the calcein-positive cells were small, atrophied, and

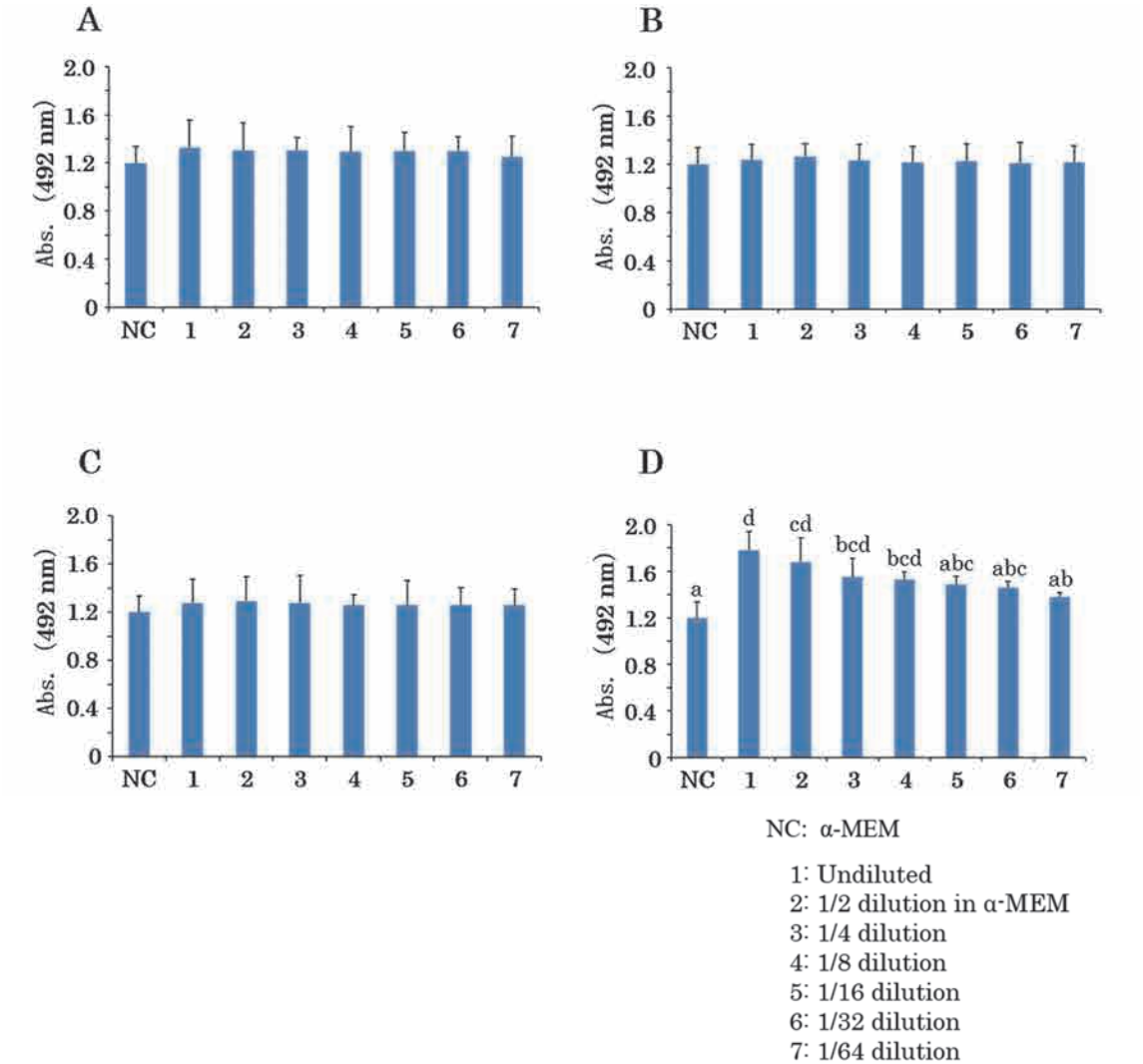

Figure 2　Evaluation of the activity of lactate dehydrogenase (LDH) released by damaged cells

A: Cement A experimental medium.

B: Cement B experimental medium.

C: Cement C experimental medium.

D: Calcium hydroxide experimental medium.

The graph shows the mean values and standard deviations for each group (n = 4).

Statistically significant differences are indicated by different letters ($p < 0.05$).

round, with minimal cell spreading (Figure 3).

3. 4 Morphological evaluation of hDPSCs in the experimental culture media

Vinculin-positive signals were observed on the tips of most hDPSCs cultured in the Cement A, B, and C media, and they appeared to have adhered to the culture dish, as was the case for the NC culture (Figure 4A, D). In particular, the hDPSCs in the Cement B medium exhibited marked cell spreading, as observed in the NC culture (Figure 4B, C). In the calcium hydroxide medium, a small number of hDPSCs showed slight spreading and almost no vinculin signal at the tip of the cells (Figure 4E). Comparative measurements of the pericellular lengths and areas of the hDPSCs cultured in each medium indicated that the hDPSCs cultured in Cement B medium had a significantly larger pericellular length and area than

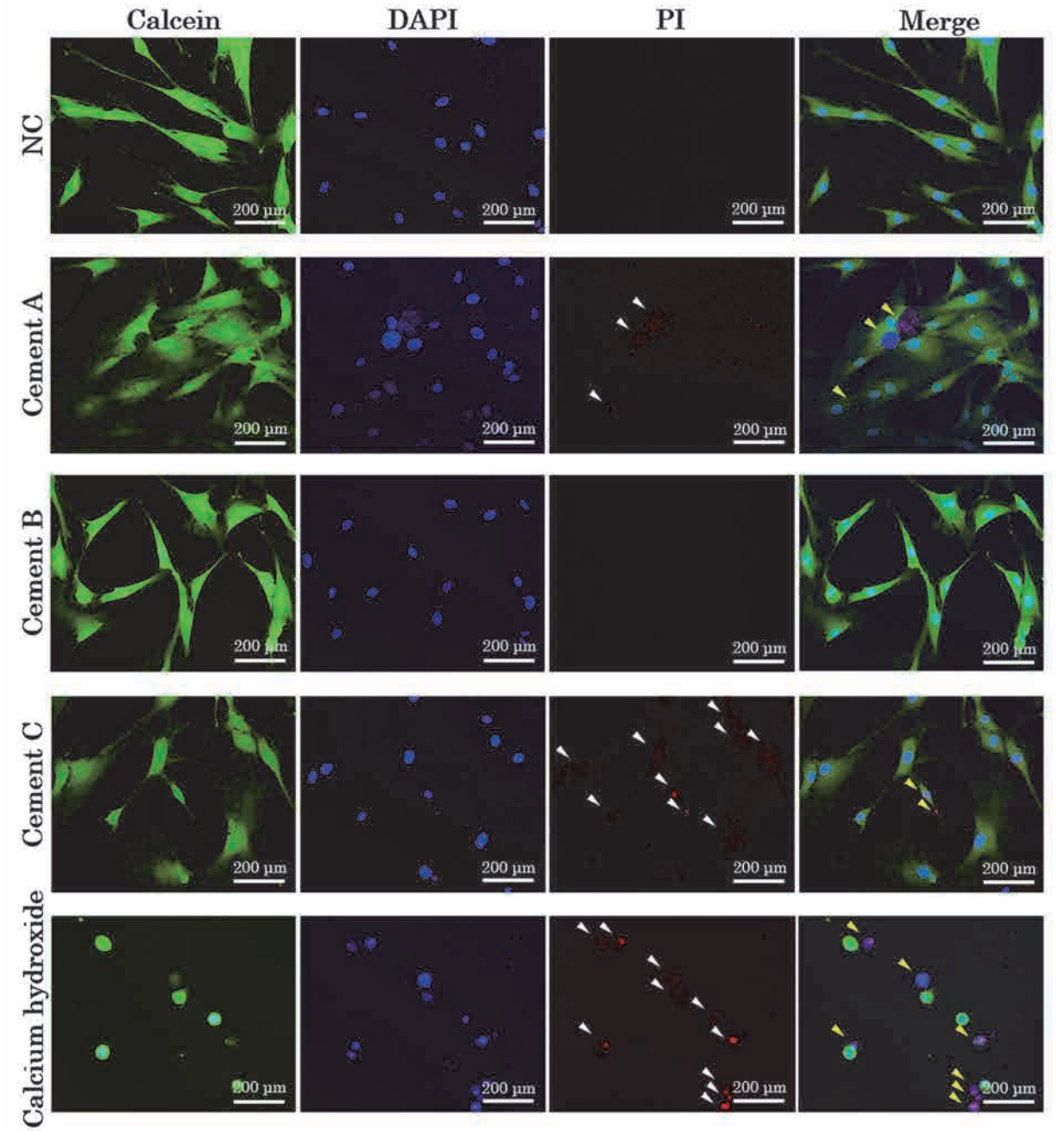

Figure 3　Live and dead determination of cells by fluorescence staining

The calcein, DAPI, and propidium iodide (PI) staining images, as well as superimposition of these images, are shown for hDPSCs (upper row) cultured with α MEM (NC) and for the Cements A, B, and C experimental culture media and medium with the eluate from calcium hydroxide cement. White arrow heads indicate PI-positive cells, and yellow arrow heads show strongly PI-positive cells.

those cultured in the other media and had the mostly similar cell morphology as in the NC medium (Figure 4F, G).

3. 5 Evaluation of cell death in the dental pulp, 7 days after pulp capping with experimental cement

A few TUNEL-positive cells were observed among the pulp tissues that had undergone pulp capping with Cement A (Figure 5A). Hardly any TUNEL-positive

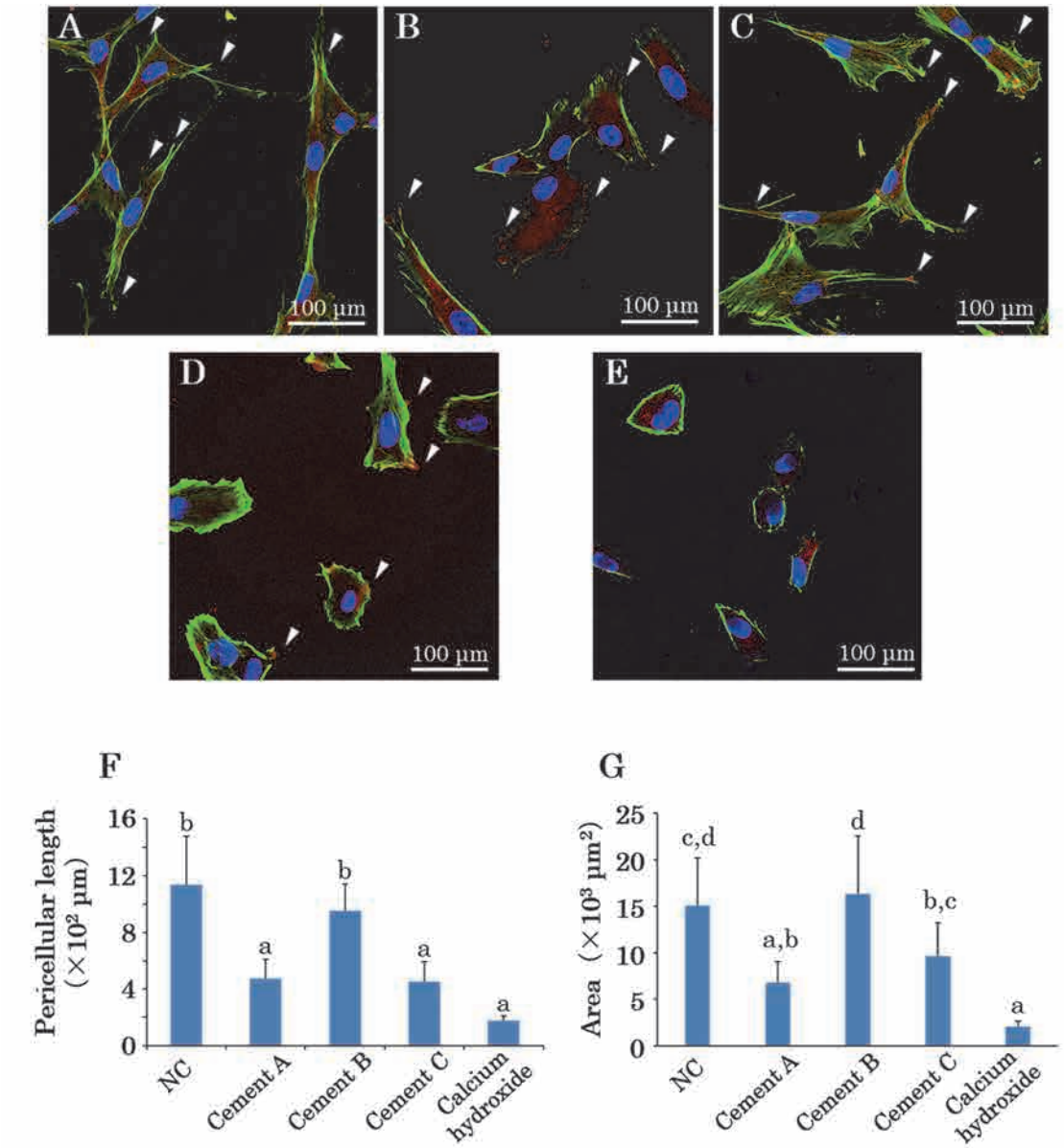

Figure 4　Fluorescent images of vinculin and actin of hDPSCs in the NC and experimental media

A: hDPSCs cultured in the NC medium.

B: hDPSCs in the Cement A experimental medium.

C: hDPSCs in the Cement B experimental medium.

D: hDPSCs in the Cement C experimental medium.

E: hDPSCs in the calcium hydroxide experimental medium.

Vinculin is shown in red (Alexa Fluor 594), actin in green (Alexa Fluor 488), and cell nuclei in blue (DAPI). The white arrow heads indicate the vinculin localization.

F: Comparison of the pericellular lengths of the hDPSCs cultured in NC and experimental media.

G: Comparison of the hDPSC area calculated from the pericellular length shown in panel F.

cells were observed in the tissue with the Cement B pulp capping (Figure 5B). In contrast, many TUNEL-positive cells were noted in the tissue with the Cement C pulp capping (Figure 5C), and markedly more TUNEL-positive cells were observed in the tissue with the calcium hydroxide pulp capping (Figure 5D).

3. 6 Evaluation of cell proliferation in the dental pulp tisssu, 7 days after pulp capping with experimental cement

Although only a few PCNA-positive cells were observed in the dental pulp tissue that had undergone the pulp capping with Cement A or C (Figure 6A, C), numerous PCNA-positive cells were observed in the tissue pulp-capped with Cement B (Figure 6B). Hardly any PCNA-positive cells were observed in the tissue with the calcium hydroxide pulp capping (Figure 6D).

4 Discussion

Calcium phosphate materials are highly biocompatible [14, 15, 17-20] and achieve favorable results

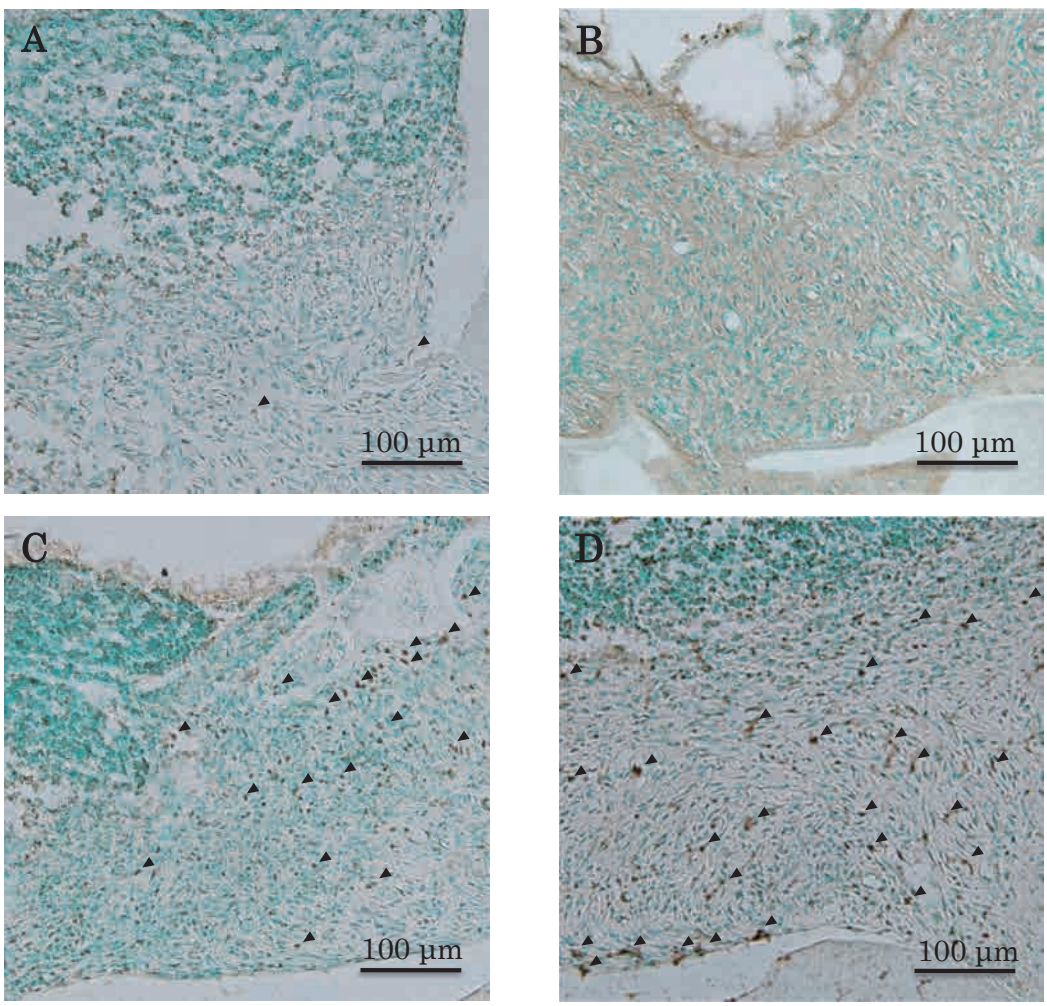

Figure 5 Distribution of TdT-mediated dUTP nick-end labeling (TUNEL) -positive cells 7 days after pulp capping.

A: TUNEL staining image of dental pulp tissue on day 7 after pulp capping with Cement A.

B: After pulp capping with Cement B.

C: After pulp capping with Cement C.

D: After pulp capping with calcium hydroxide.

The black arrow heads show TUNEL-positive cells.

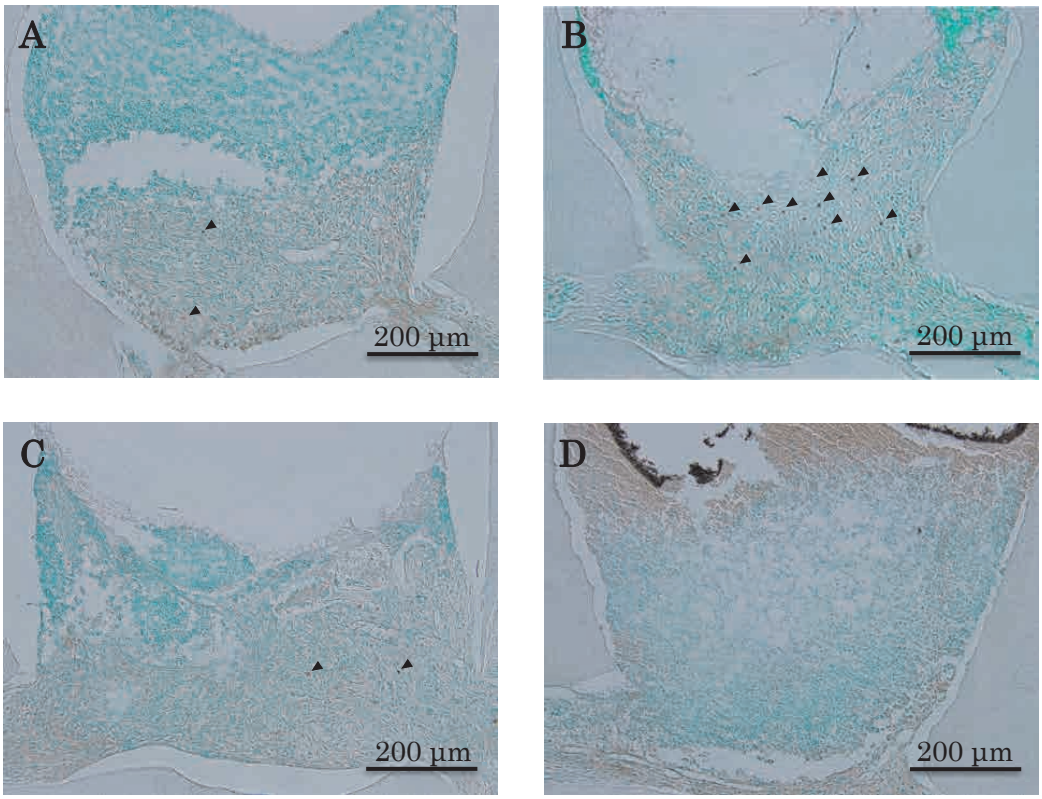

Figure 6　Proliferation cell nuclear antigen (PCNA) staining images 7 days after pulp capping.
A: PCNA staining image of pulp tissue image of dental pulp tissue on day 7 after pulp capping with Cement A.
B: After pulp capping with Cement B.
C: After pulp capping with Cement C.
D: After pulp capping with calcium hydroxide.
The black arrow heads show PCNA-positive cells.

when used as bone replacement materials [14, 27, 28]. In this study, we evaluated our two-component calcium phosphate materials, α-TCP /Te-CP, using three different mixing conditions. Eight different mixtures were used in previous studies, and the aqueous solutions of sodium dihydrogen phosphate and citric acid, which showed positively dentin bridge formation, were employed in the present study. We used an hDPSC culture system to compare the results obtained with these cements with those of a conventional and widely used calcium hydroxide preparation. hDPSC proliferation was not inhibited by any of the three α-TCP/Te-CP cements, and no clear cytotoxicity was observed. However, cell morphology showed differences among with the three types of α-TCP /Te-CP cement. This result suggested to be related to leached Ca and P. In particular, calcium ions are one of the important cellular second messenger, and high concentrations of Ca have been reported to affect cellular responses[29-31]. The calcium hydroxide markedly inhibited the hDPSC proliferation and strong LDH activity was observed. Many studies have shown that calcium hydroxide preparations irritate the dental pulp [7, 9-11]. In the present study, we also observed that calcium hydroxide damaged hDPSCs in the culture system. Because this damage was reduced by diluting the culture, cell proliferation inhibition and cytotoxicity appeared to be dependent on the components eluted from the calcium hydroxide. However, calcium hydroxide has shown the ability to form dentin bridges *in vivo*, and some reports supported the efficacy of alkaline stimulation on dentin bridge formation.

In a previous report, we found that the three types

of α-TCP/Te-CP cements used in the present study showed the same hard tissue induction ability in the rat lined medullary model as obtained with calcium hydroxide. However, no significant differences in tissue response after pulp capping were observed among the three types of α-TCP/Te-CP cement[23]. Therefore, in this study, the reactivity of hDPSCs with cements A, B, and C was analyzed in detail. Few PI-positive cells were observed in hDPSCs cultured in Cement B medium. However, some of these cells were present in Cement A medium. Relatively more PI-positive cells were observed in cement C medium, followed by calcium hydroxide medium. No differences in cement properties were found between the different cement mixtures used. Similar to calcium hydroxide cement, a large number of PI-positive cells were observed in the result of cement C, which does not contain P in the eluted component, suggesting that the balance between Ca and P was also involved in the effect on the cell activities. Histochemical analysis in the rat exposure model also showed that many proliferating cells and few TUNEL-positive cells were observed in pulp tissue covered with cement B, whereas few proliferating cells were observed in tissue covered with cements A and C. A large number of TUNEL-positive cells were observed in tissue covered with cement C. These results supported the importance of the balance between Ca and P eluted from cement materials.

The primary objective of this study was to verify the applicability of our previously developed one-step sintering method for a two-component calcium phosphate α-TCP/Te-CP cement as a pulp capping material, and we showed that this objective has been achieved based on the results of this study. Evaluation of the cell culture system and histological evaluation showed that Cement B has proper biocompatibility. It was also shown that the same cement powder with different mixing solutions produced different cellular and tissue responses. However, the mixing solutions used in this study did not have the same conditions for phosphoric acid content, and the experimental system could not be simply compared due to the presence of multiple parameters. Further analysis should evaluate the performance of experimental cement materials as a pulp capping agent with the limited parameters for comparison, such as using phosphate buffers with several different pH adjustments.

5 Conclusion

Our results showed that α-TCP/Te-CP, molar ratio 1/2, cement mixtured with 2 M sodium dihydrogen phosphate solution exhibited satisfactory cellular reactivity and pulp tissue affinity. These findings indicate that α-TCP/Te-CP cement prepared by our one-step sintering process was a promising pulp capping agent.

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