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Functional evaluation of mineral trioxide aggregate cement with choline dihydrogen phosphate

Kazuhisa TABIRA[1,2], Noboru KAJIMOTO[1], Hirogo MINAMISAWA[1], Taira SATO[3], Michito MARUTA[1], Kyoko OKA[2,4], Takuya KATAOKA[5], Tomohiko YOSHIOKA[5], Satoshi HAYAKAWA[5] and Kanji TSURU[1]

[1] *Section of Bioengineering, Department of Dental Engineering, Fukuoka Dental College, Fukuoka 814-0193, Japan*
[2] *Section of Pediatric Dentistry, Department of Oral Growth and Development, Fukuoka Dental College, Fukuoka 814-0193, Japan*
[3] *Section of Biomaterials, Department of Dental Engineering, Fukuoka Dental College, Fukuoka 814-0193, Japan*
[4] *Oral Medicine Research Center, Fukuoka Dental College, Fukuoka 814-0193, Japan*
[5] *Biomaterials Laboratory, Faculty of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama 700-8530, Japan*
Corresponding author, Noboru KAJIMOTO; E-mail: kajimoto@fdcnet.ac.jp

To improve the cytocompatibility of mineral trioxide aggregate (MTA) cement and its ability for reparative dentin formation, the effect of adding choline dihydrogen phosphate (CDHP), which is reported to be biocompatible, to MTA cement was investigated. The L929 cell proliferation showed that the addition of CDHP improved cell viability. The addition of CDHP shortened the setting time of MTA cement, with a significant decrease in consistency above 0.4 g/mL. Diametral tensile strength of the set cement was improved by the addition of 0.4 g/mL CDHP. Solubility was judged to be within the range of clinical application. The spontaneous precipitation of low crystalline hydroxyapatite was examined by immersing the set cement in phosphate buffer saline, and it was found that the ability of the cement with 0.4 g/mL of CDHP was significantly improved compared with that of the cement without CDHP.

Keywords: Mineral trioxide aggregate (MTA), Choline dihydrogen phosphate (CDHP), Cytocompatibility, Low crystalline hydroxyapatite, Reparative dentin

INTRODUCTION

Mineral trioxide aggregate (MTA) cement is a hydraulic cement modified from portland cement, a civil engineering and construction cement, for dental use[1-6]. It has become popular as a cement that can be used to treat difficult endodontic conditions, such as direct pulp lining and root canal wall perforation sealing, by forming new hard tissue[7-9]. The chemical composition of MTA cement is composed of portland cement as the main ingredient, plus bismuth oxide to enhance radiopacity, and a small amount of gypsum. The most representative MTA cement, PROROOT®MTA (DENTSPLY, Johnson City, TN, USA), has also been subjected to crystalline phase identification[10]. The setting reaction is mainly a hydration reaction of tricalcium silicate and dicalcium silicate, which produces insoluble calcium silicate hydrate and precipitates calcium hydroxide crystals during setting reaction[11,12]. It has been reported that the precipitated calcium hydroxide reacts slowly with body fluids to deposit apatite-like calcium phosphate salts on the cement surface, and this mechanism is thought to contribute to the biocompatibility and sealing properties of MTA cements[13-15]. However, it has also been reported that MTA cements exhibit cytotoxicity in the early stages of setting[16]. Considering that dentin cells and other cells contribute to the formation of reparative dentin in MTA cements, it is crucial to enhance the cytocompatibility of the cement in the early stages of setting, and the addition of components that enhance the ability to form reparative dentin may be effective.

To improve the functionality of MTA cements, especially the ability to form reparative dentin, we first attempted to improve cell proliferation and apatite-like calcium phosphate precipitation in the early stages of setting. The substance focused on in this study is choline dihydrogen phosphate (CDHP; Fig. 1), which is used as an ionic liquid composed of biogenic choline and phosphate ion, The biocompatibility of CDHP has been reported by Elliott *et al.*[17,18]. Another advantage is that it can be mixed with MTA cement due to high water solubility. Furthermore, orthophosphate ion in the CDHP structure is expected to contribute sufficiently to apatite-like calcium phosphate precipitation on the surface of MTA cements because the orthophosphate ion is a chemical component of hydroxyapatite.

In this study, various concentrations of CDHP were added to commercial MTA cements to investigate whether the addition of CDHP could overcome the cytotoxicity in the early stages of cement setting, and to investigate the effects of the addition of CDHP on various basic properties of the cements, such as setting time and consistency. In addition, the effect of CDHP addition on the calcium phosphate precipitation ability of MTA cement was also investigated.

MATERIALS AND METHODS

Cytocompatibility of MTA cement with CDHP in the early stage of setting

1. Preparation of MTA cement extract

First, a cementing solution containing CDHP (Kanto Kagaku, Tokyo, Japan) was prepared by dissolving CDHP in ultrapure water (FUJIFILM Wako Pure

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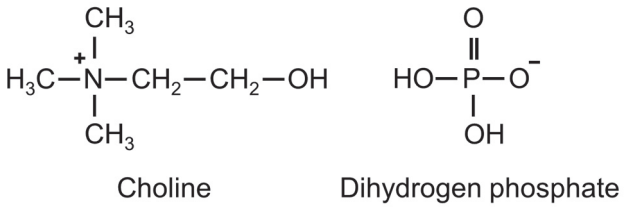

Fig. 1 Chemical structural formula of CDHP.

Chemical, Osaka, Japan) and adjusting to pH 7.0 with sodium hydroxide solution (FUJIFILM Wako) to obtain neutral CDHP solutions of 0, 0.2, 0.4, 0.6, and 0.8 g/mL. The method for preparing the extract from cement pasteurized mud was adapted from Wadajkar *et al.*[16]. Commercially available PROROOT®MTA (DENTSPLY) was used as MTA cement, and PROROOT®MTA powder was mixed with the aforementioned neutral CDHP solution at the powder/liquid (P/L) ratio of 0.5/0.18, as described in the instruction manual, to obtain cement pasteurized mud. A 0.2 g of cement mud was added immediately after kneading was added into 2.0 mL of the medium used for cell proliferation tests (see below for details), and allowed to stand for 6 h in a 5% CO_2 incubator at 37°C. The supernatant was used as the extract for subsequent experiments.

2. Cell proliferation test and cell morphology observation
L929 fibroblasts (RIKEN BioResource Research Center, Tsukuba, Japan) were used in this study. Cells were grown in minimum essential medium eagle (MEM; SIGMA-ALDRICH, St Louis, MO, USA) containing 10% fetal bovine serum (FBS; GIBCO Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (FUJIFILM Wako). Cell suspensions were adjusted to 2.5×10^4 cells/mL, seeded at 0.1 mL/well in 96-well plates, and incubated in a 5% CO_2 incubator at 37°C (ASTEC, Fukuoka, Japan) for 24 h. Then the cell culture medium was removed and 0.1 mL/well of the extracts described above was added. For the blank, 0.1 mL/well of cell culture medium was added instead of the extractant. The number of cells after 24 h was evaluated using an MTT cell count kit (Nacalai Tesque, Kyoto, Japan). The absorbance of the extract and the blank were measured at a test wavelength of 570 nm and reference wavelength of 650 nm on a microplate reader (Awareness Technology, Palm City, FL, USA), and the cell viability was derived from the following formula. The mean value and standard deviation were calculated with n=6.

Cell viability (%)=absorbance of extract/absorbance of blank×100

Cell morphology after 24 h culture was observed and photographed with a digital camera (Full HD HDMI Camera TrueChromeII Plus, Tucsen Photonics, Foochow, China) attached to a trinocular inverted phase contrast microscope (TBI, YASHIMA OPTICAL, Saitama, Japan).

Initial setting time of MTA cement with CDHP
The initial setting time was measured with reference to JIS T6609-1:2015 (Dentistry: Water-based cements)[19]. Cement mud was prepared using PROROOT®MTA (DENTSPLY) at a P/L ratio of 0.5/0.18 with five different mixing solutions (0, 0.2, 0.4, 0.6, and 0.8 g/mL CDHP). The obtained mud was filled into a silicone mold (6 mm diameter×3 mm height) and cured at 37°C under 100% relative humidity. Vicar needle was dropped on the cement mud every 15 min from the start of kneading, and the time required for no indentation marks to be left behind was taken as the initial setting time. The mean and standard deviation were determined with n=4.

Consistency evaluation of MTA cement with CDHP
The consistency was evaluated with reference to JIS T6522:2015 (Dental root canal sealing materials)[20]. The cement mud was prepared using three different mixing solutions (0, 0.2, and 0.4 g/mL) with different CDHP contents at a P/L ratio of 0.5/0.18. Immediately after kneading, the cement mud was filled into a syringe (TERUMO Syringe 1 mL, TERUMO, Tokyo, Japan) and 0.05 mL was extruded onto a glass plate. A 120 g weight was placed on the plate 180 s after the start of kneading and then removed 10 min after the start of kneading. Photographs of weight-compressed cement mud on plate were taken with a stereomicroscope (LeicaS9i, Leica Microsystems, Wetzlar, Germany). The photographed images were binarized with the "autothreshold" function using image analysis software (ImageJ 1.52a), and the maximum and minimum diameters of the cement compacted into disks were calculated with "Analyze Particles" command. The average of the maximum and minimum diameters was calculated as the consistency. The test was performed with n=3.

Diametral tensile strength of MTA cement with CDHP
Cement mud was prepared using PROROOT®MTA (DENTSPLY) at a P/L ratio of 0.5/0.18 with five different mixing solutions (0, 0.2, 0.4, 0.6, and 0.8 g/mL CDHP). The obtained mud was filled into split stainless-steel molds (6 mm diameter×3 mm height) and cured at 37°C under 100% relative humidity. After 24 h from the start of reaction, the set cement was immersed in isopropyl alcohol (FUJIFILM Wako) to stop the setting reaction. The mechanical strength of the set cements was evaluated in the form of diametral tensile strength (DTS). After drying, the diameter and height of each specimen were measured using a micrometer (MDC-25MU, Mitutoyo, Kanagawa, Japan). A load was applied to crush each specimen in a universal testing machine (AGS-J, Shimadzu, Kyoto, Japan) at a crosshead speed of 10 mm/min. The mean and standard deviation were determined with n=5.

Solubility (%) of MTA cement with CDHP
Solubility (%) was measured with reference to JIS T6522:2015 (Dental root canal sealing materials)[20]. A Teflon ring (20 mm inner diameter and 1.5 mm height) was placed on a plastic sheet placed on a glass plate

and filled with 2.0 g of cement mud prepared by the method described above. A glass plate with another pair of plastic sheets was pressed onto the cement mud and then cured for 24 h at 37°C under 100% relative humidity. The set cements were removed from the ring and immersed in isopropyl alcohol to stop the setting reaction. After drying thoroughly, the specimens were weighed. Two specimens were placed in a glass petri dish and 50 mL of ultrapure water was added. The petri dishes were covered and kept at 37°C under 100% relative humidity for 24 h. The solution was filtered and placed in a thermostatic bath set at 110°C to evaporate the water, and the weight of the residue was measured until a certain weight was reached. The solubility (%) was determined from the weight before the test and the weight of the residue. The mean and standard deviation were determined with n=3.

Evaluation of calcium phosphate precipitation on MTA cement with CDHP

PROROOT®MTA (DENTSPLY) was kneaded at a P/L of 0.5 g/0.18 mL and filled into a silicone mold with a hole 5 mm diameter and 1 mm height. The mold with cement was then immediately cured at 37°C under 100% relative humidity. After 24 h, set cements were removed from mold, and further setting reaction was stopped with isopropyl alcohol. After the alcohol has completely evaporated, the set cements were immersed in ultrapure water or Ca- and Mg-free phosphate buffer saline (PBS; Dulbecco's Phosphate Buffered Saline; GIBCO Thermo Fisher Scientific) at 37°C for 24 h based on the report of Han *et al.*[14]. The specimens were then rinsed with a plenty of ultrapure water and dried in an oven at 60°C before being used in subsequent evaluations. Surface observation by scanning electron microscopy (SEM; JCM-6000 Plus, JEOL, Tokyo, Japan) and elemental analysis by an energy dispersive X-ray (EDX) microanalyzer (Genesis APEX2, AMETEK, Paoli, PA, USA) were performed to examine calcium phosphate precipitation by immersion in each solution. The samples for SEM were coated with Au using a magnetron sputterer (DII-29010SCTR Smart Coater, JEOL) and the samples for EDX were coated with carbon (VC-100S/W, VACUUM DEVICE, Ibaraki, Japan) before analyses. SEM observations were performed at 15 kV while EDX analysis was done at 10 kV in an accelerating voltage. For EDX analysis, five different locations of the same sample were analyzed at 1,000× magnification to select O, Si, P, and Ca —the major components of MTA cement and calcium phosphate precipitation— and to determine the percentage of each element with the sum of these elements as 100%. The mean and standard deviation were determined with n=5. In addition, thin-film X-ray diffraction (TF-XRD; X'Pert-ProMPD, PANalytical, Almelo, the Netherlands: CuKα, λ=0.15418 nm, 45kV-40mA) was used to identify the crystalline phase, where all data were collected in the step-scan mode (step size: 0.02° from 20° to 40° in 2θ, counting time: 8 s per step) with a constant X-ray incident angle θ of 1.0°.

Statistical analysis

Statistical analysis was performed with statistical software (R version 4.0.3, R Foundation for Statistical Computing, Vienna, Austria). One-way analysis of variance (ANOVA) and Tukey's multiple-comparison test were used to analyze the data (p<0.05).

RESULTS

Figure 2 shows the viability of L929 cells after 24 h of incubation in medium containing components extracted from MTA cement kneading mud. Cell viability of the CDHP-added systems (0.2CDHP, 0.4CDHP, 0.6CDHP, and 0.8CDHP) was significantly higher than that of the control without CDHP (0CDHP). There was no significant difference in cell viability in the range of 0.2 to 0.8 g/mL CDHP. Figure 3 shows the morphology of L929 cells after 24 h of culture in medium containing components extracted from MTA cement kneading mud. Spherical cells were observed when cultured in MTA cement extraction medium with no CDHP added (0CDHP). However, more spindle-shaped cells were observed in the CDHP-added systems, and no difference in cell morphology was observed in the CDHP concentration range of 0.2 to 0.8 g/mL.

The initial setting time of the MTA cement mud without CDHP (0CDHP) and with different concentrations of CDHP (0.2CDHP, 0.4CDHP, 0.6CDHP, and 0.8CDHP) is shown in Fig. 4. The initial setting time of 0CDHP was about 6 h while those of the CDHP-added systems were about 3 h, indicating a shorter setting time resulting from the addition of CDHP. There was no significant difference in initial setting time at CDHP concentrations ranging from 0.2 to 0.8 g/mL. The consistency of MTA cement mud without CDHP (0CDHP) and with different concentrations of CDHP (0.2CDHP and 0.4CDHP) is shown in Fig. 5. Addition of CDHP

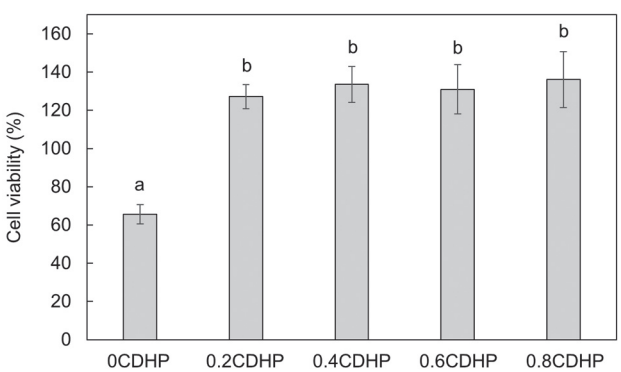

Fig. 2 L929 cells viability after 24 h of culture in medium containing components extracted from MTA cement mud without and with different concentrations of CDHP.
Same lowercase letters indicate no statistically significant differences while different lowercase letters indicate significant differences (n=6, p<0.05).

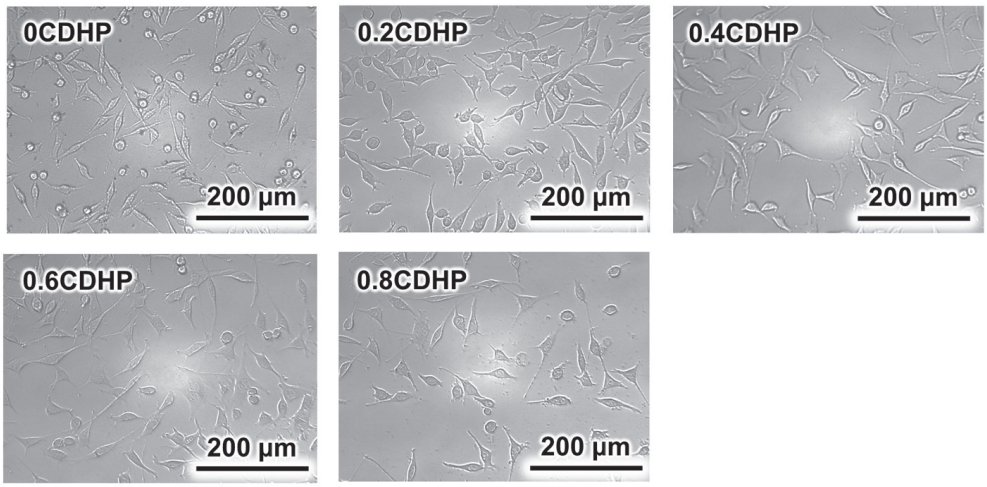

Fig. 3 L929 cells morphology after 24 h of culture in medium containing components extracted from MTA cement mud without and with different concentrations of CDHP.

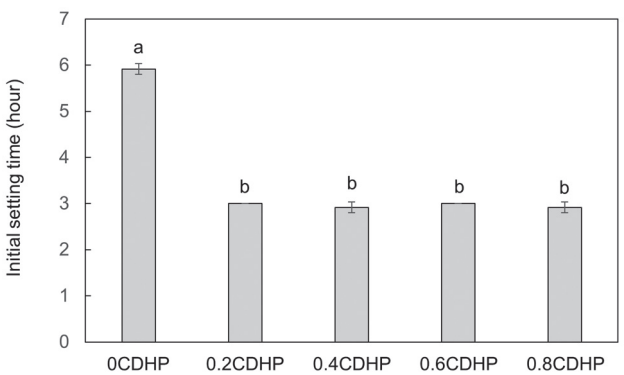

Fig. 4 Initial setting time of MTA cement mud without and with different concentrations of CDHP.
Same lowercase letters indicate no statistically significant differences while different lowercase letters indicate significant differences (n=4, p<0.05).

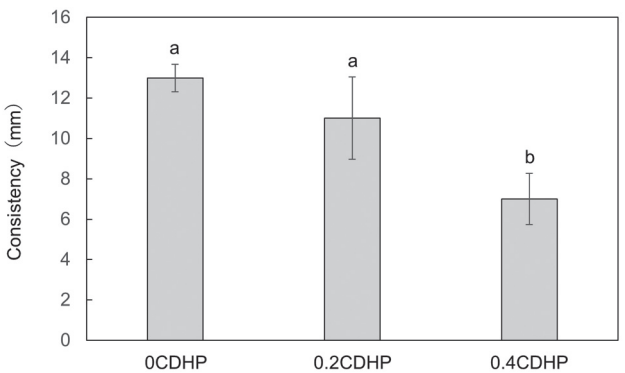

Fig. 5 Consistency of MTA cement mud without and with different concentrations of CDHP.
Same lowercase letters indicate no statistically significant differences while different lowercase letters indicate significant differences (n=3, p<0.05).

up to 0.2 g/mL did not affect the consistency of MTA cement mud, but the consistency of cement mud with 0.4 g/mL of CDHP significantly decreased. Furthermore, when more than 0.6g/mL of CDHP was added to the cements, the cement mud could not be pushed out from the syringe, and the consistency could not be evaluated in the range from 0.6 to 0.8 g/mL. Figure 6 shows DTS of set MTA cement with different concentrations of CDHP (0CDHP, 0.2CDHP, 0.4CDHP, 0.6CDHP, and 0.8CDHP) after being cured for 24 h at 37ºC under 100% relative humidity. The set cement with 0.4 g/mL CDHP (0.4CDHP) had significantly higher value than that of the set cement without CDHP (0CDHP). Furthermore, as the concentration of CDHP was increased from 0.4 to 0.8 g/mL, the DTS values tended to decrease. Figure 7 shows the solubility (%) of set MTA cements without CDHP (0CDHP) and with CDHP (0.2CDHP and 0.4CDHP) after being cured for 24 h at 37ºC under 100% relative humidity. Although the solubility (%) of set MTA cements was slightly increased by the addition of CDHP, there was no significant difference in the solubility between 0.2CDHP and 0.4CDHP. The values of 0.2CDHP and 0.4CDHP were less than 3% and within the criteria for dental root canal sealing materials[18].

Figure 8 shows SEM images of set MTA cement without CDHP (0CDHP), with 0.2 g/mL CDHP (0.2CDHP) and 0.4 g/mL CDHP (0.4CDHP) after being cured for 24 h at 37ºC under 100% relative humidity, and followed by immersion in ultrapure water (0CDHP_UW, 0.2CDHP_UW, and 0.4CDHP_UW) or PBS (0CDHP_PBS, 0.2CDHP_PBS, and 0.4CDHP_PBS) for 24 h at 37ºC. A typical MTA cement surface, similar to that reported by Lee *et al.*[21], was observed before immersion in ultrapure water or PBS. There was no obvious difference between

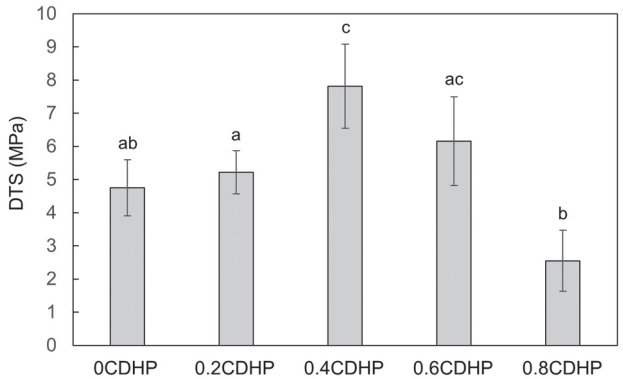

Fig. 6 DTS of set MTA cements with different concentration of CDHP cured for 24 h at 37°C under 100% relative humidity.
Same lowercase letters indicate no statistically significant differences while different lowercase letters indicate significant differences (n=5, p<0.05).

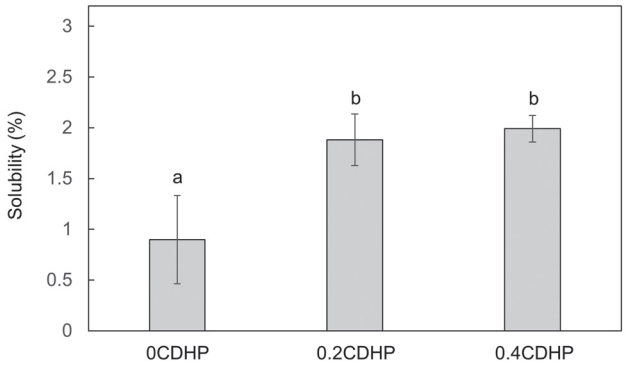

Fig. 7 Solubility (%) of set MTA cements without CDHP and with 0.2 mg/mL CDHP cured for 24 h at 37°C under 100% relative humidity Same lowercase letters indicate no statistically significant differences while different lowercase letters indicate significant differences (n=3, p<0.05).

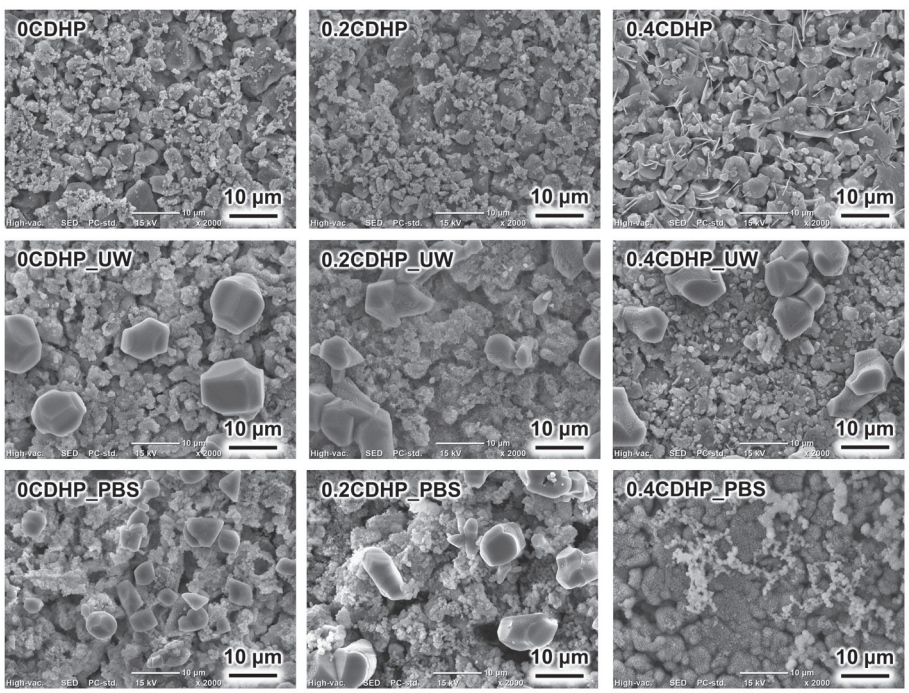

Fig. 8 SEM images of set MTA cement without CDHP (0CDHP), with 0.2 g/mL CDHP (0.2CDHP) and 0.4 g/mL CDHP (0.4CDHP) cured for 24 h at 37°C under 100% relative humidity, and followed by the immersion in ultrapure water (0CDHP_ UW, 0.2CDHP_UW, and 0.4CDHP_UW) or PBS (0CDHP_ PBS, 0.2CDHP_ PBS, and 0.4CDHP_PBS) for 24 h at 37°C.

the surfaces of 0CDHP and 0.2CDHP, but plate-like substances about 5 microns in size were observed on 0.4CDHP. 0CDHP_UW (0CDHP immersed in ultrapure water) showed a clear change compared with the surface before immersion. In addition, precipitations about 5–10 microns in size were also observed. 0CDHP_PBS (0CDHP immersed in PBS) showed an increase in surface reaction and more precipitates were observed. The surface of 0.2CDHP_UW was similar to 0CDHP_UW and the surface of 0.2CDHP_PBS was similar to 0CDHP_PBS, but the 0.2CDHP system showed more advanced surface reaction and more precipitates than the 0CDHP system. More changes were observed for 0.4CDHP. The plate-like precipitates on 0.4CDHP were not observed after

immersion in ultrapure water or PBS. In particular, the cement surface was observed to be completely covered with a layer consisting of petal-like precipitates after PBS immersion. The chemical composition ratios (atomic %, O:Si:P:Ca) of the surfaces of set MTA cement without CDHP (0CDHP), with 0.2 g/mL CDHP (02CDHP), and with 0.4 g/mL CDHP (04CDHP) after 24 h of curing at 37°C under 100% relative humidity, and followed by the immersion in ultrapure water or PBS for 24 h are shown in Table 1. The addition of CDHP to MTA cement increased the percentage of P as the amount of CDHP added was increased. In case of 0CDHP, an increase in the percentage of P was observed after immersion in PBS. For 0.2CDHP, the percentage of P decreased after immersion in ultrapure water whereas it was increased after immersion in PBS. For 0.4CDHP, the percentage

Table 1 Chemical composition (atomic %) of set MTA cement without CDHP (0CDHP), with 0.2 g/mL CDHP (0.2CDHP) and 0.4 g/mL CDHP (0.4CDHP) cured for 24 h at 37ºC under 100% relative humidity, and followed by the immersion in ultrapure water (0CDHP_UW, 0.2CDHP_UW, and 0.4CDHP_UW) or PBS (0CDHP_ PBS, 0.2CDHP_PBS, and 0.4CDHP_PBS) for 24 h at 37ºC

Sample codes	Composition (at%)			
	O	Si	P	Ca
0CDHP	69.2±3.7	10.1±0.5	0.0±0.0	20.6±3.5
0.2CDHP	72.5±3.0	9.6±0.4	1.2±0.1	16.5±3.1
0.4CDHP	64.5±0.2	8.8±0.2	2.0±0.1	24.4±0.2
0CDHP_UW	64.8±1.7	12.5±0.8	0.0±0.0	22.4±0.4
0.2CDHP_UW	64.3±0.3	11.1±0.5	0.9±0.1	23.5±0.5
0.4CDHP_UW	66.0±0.6	9.1±0.4	2.1±0.1	22.6±0.5
0CDHP_PBS	64.4±0.8	11.0±0.3	1.5±0.2	22.9±0.8
0.2CDHP_PBS	64.5±1.9	8.3±0.8	3.8±0.9	23.3±1.2
0.4CDHP_PBS	58.8±0.7	0.4±0.1	15.1±0.2	25.5±0.4

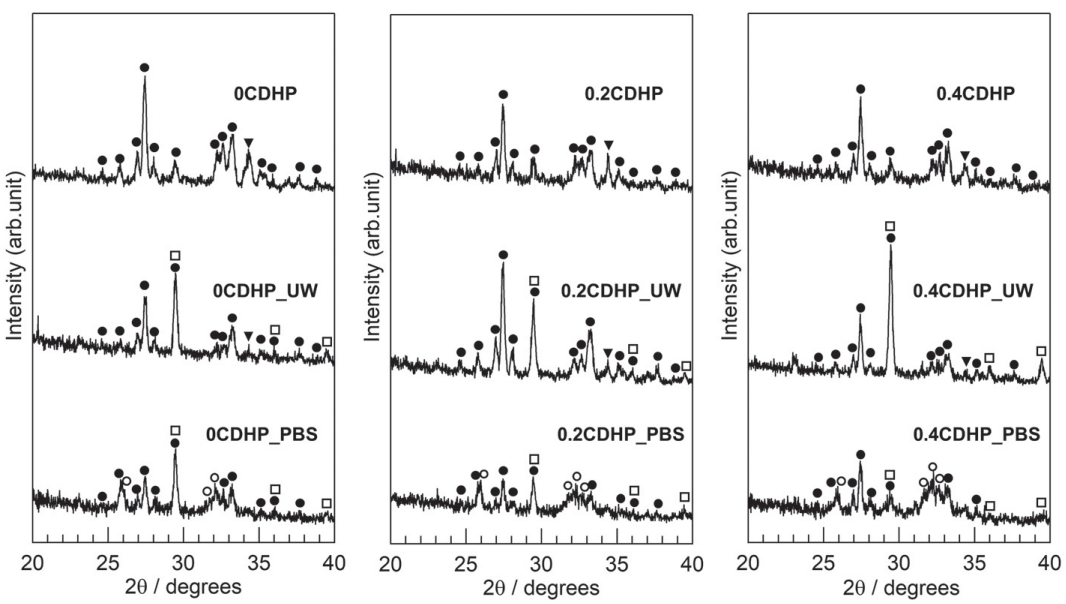

Fig. 9 TF-XRD patterns of set MTA cement without CDHP (0CDHP), with 0.2 g/mL CDHP (0.2CDHP) and 0.4 g/mL CDHP (0.4CDHP) cured for 24 h at 37ºC under 100% relative humidity, and followed by the immersion in ultrapure water (0CDHP_UW, 0.2CDHP_UW, 0.4CDHP_UW) or PBS (0CDHP_ PBS, 0.2CDHP_PBS, 0.4CDHP_PBS) for 24 h at 37ºC.
●: set MTA cement, ▼: calcium hydroxide (PDF#44-1481) □: calcite (PDF#5-586), ○: hydroxyapatite (PDF#9-432)

of P changed little after immersion in ultrapure water, but the percentage of P increased significantly after immersion in PBS. Figure 9 shows TF-XRD patterns of set MTA cement without CDHP (0CDHP), with 0.2 g/mL CDHP (0.2CDHP) and 0.4 g/mL CDHP (0.4CDHP) cured for 24 h at 37ºC under 100% relative humidity, and followed by the immersion in ultrapure water (0CDHP_UW, 0.2CDHP_UW, 0.4CDHP_UW) or PBS (0CDHP_ PBS, 0.2CDHP_PBS, 0.4CDHP_PBS) for 24 h at 37ºC. Before soaking in ultrapure water or PBS, the XRD patterns consisted of diffraction peaks derived from MTA components and curing products including calcium hydroxide ($Ca(OH)_2$; PDF#44-1481), and almost no differences were observed with or without CDHP. In addition to these peaks, a new diffraction peak assigned to calcite ($CaCO_3$; PDF#5-586) was detected on the surface after immersion in ultrapure water. On the other hand, not only calcite, but also hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; PDF#9-432) were detected on set MTA cements with or without the addition of CDHP after immersion in PBS. Although the peaks assigned to hydroxyapatite were broad, the intensity increased with increasing amount of CDHP.

DISCUSSION

In this study, we investigated the effect of adding CDHP, which has been reported to have biocompatibility, to MTA cements to improve their cytocompatibility in the early stages of setting and to enhance their ability to form reparative dentin.

The cell viability of MTA cement (0CDHP) was less than 100% (Fig. 2), which is consistent with previous reports[16], and the lower cell viability may be due to the alkaline pH of MTA cement immediately after mixing. This is supported by the observation herein of spherical cell morphology (Fig. 3), and the increased cell viability of 120–130% in all the systems in which CDHP was added to the MTA cement. This result may be due to the effect of the CDHP being added to the MTA cement. L929 cells were observed to be elongated into a spindle shape with a size of about 100 μm, which is a typical morphology of L929 as an adhesive cell[22]. Elliott *et al.*[17,18] also reported a decrease in cell viability above a certain level with increasing CDHP concentrations, but no decrease in cell viability was observed in the 0.2–0.8 g/mL range in the present study. The reason for this difference is presumably that the amount of CDHP dissolved from the cement into the culture medium was small and did not reach a concentration that would decrease the cell viability. The reason is presumably also the pH of the cell culture medium. They added CDHP directly to the culture medium, whereas we evaluated it using cement mud extract cultures. Because dissolving CDHP makes the solution acidic, we neutralized the cementing solution containing CDHP and used that.

The reason why the initial setting time was shortened by the addition of CDHP (Fig. 4) might be due to the decrease in the ratio of water to powder (increase in P/L ratio) caused by the retention of water by the added CDHP. This is also supported by the fact that the addition of CDHP decreased the consistency of the cement mud (Fig. 5). The strength of the set 0.4CDHP cement was greater than that of the set 0CDHP cement (Fig. 6), possibly due in part to the increase in the P/L ratio as the CDHP was added. The strength of the set cements decreased when the added CDHP was higher than 0.4 g/mL (Fig. 6), possibly due to the additive inhibiting the formation of the cement matrix. The addition of CDHP to MTA cement is thought to increase the solubility because CDHP is water-soluble itself (Fig. 7). However, the addition of 0.2 and 0.4 g/mL of CDHP to MTA cement was found to be acceptable for clinical application because JIS requires that the solubility of MTA cement as dental root canal sealing materials is less than 3%[20].

Next, we discuss the crystalline phases of precipitates on the surface of set MTA cements when immersed in ultrapure water or PBS. It has been reported that MTA cement forms calcium hydroxide during the setting process[11,12], and diffraction patterns of calcium hydroxide were detected on the set cements before immersion in ultrapure water or PBS (Fig. 9). Comparing the peak intensity of calcium hydroxide among 0CDHP, 0.2CDHP, and 0.4CDHP, it appears that the addition of CDHP suppresses the formation of calcium hydroxide, but this is a subject for future study, and the present study focuses on the crystalline phase of precipitates. The results that calcium hydroxide and calcium carbonate were detected on the set MTA cement immersed in ultrapure water are consistent with the report by Han *et al.*[14]. The peak intensity of calcium hydroxide decreased by immersion in ultrapure water, and a new diffraction peak of calcium carbonate (calcite) was detected, suggesting that calcium hydroxide was carbonated by carbonate ions present in the ultrapure water to form calcite. Since 0.4CDHP has phosphate ion derived from CDHP, it was considered that calcium phosphate could be precipitated even after immersion in ultrapure water without phosphate ion, but from the results of EDX and TF-XRD measurements (Table 1, Fig. 9), it was concluded that hydroxyapatite was not precipitated even after immersion of 0.4CDHP for 24 h in ultrapure water. On the other hand, low crystalline hydroxyapatite was detected on the surface of set MAT cement when immersed in PBS (Fig. 9). Han *et al.* concluded that amorphous calcium phosphate was precipitated by immersion in PBS since no XRD peaks assigned to hydroxyapatite was detected by XRD[14], but according to the results of this study using thin-film X-ray diffraction, which can detect only the surface, it seems reasonable to conclude that low crystalline hydroxyapatite is deposited when set MTA cement is immersed in PBS for 24 h. The amount of low crystalline hydroxyapatite precipitated increased as the amount of CDHP increased (Table 1, Figs. 8 and 9), indicating that the ability of MTA cement to spontaneously precipitate low crystalline hydroxyapatite in PBS increased with CDHP addition. It has also been reported that Ca ions supplied by the reaction of calcium hydroxide with

bodily fluids contribute to the precipitation of calcium phosphate[13-15]. However, the solubility of calcium hydroxide is extremely low, limiting the amount of calcium ions that can be leached in the bodily fluid. However, in this study, the addition of CDHP containing orthophosphate to MTA cement resulted in the leaching of phosphate ions from the cement. This effect increased the degree of supersaturation of hydroxyapatite on the cement surface, which may have facilitated heterogeneous nucleation of hydroxyapatite on the cement surface[23]. Once nuclei were formed on the cement, the crystals would have spontaneously grown to a layer by consuming calcium and phosphate ions from the surrounding solution. The relationship between the leaching behavior of phosphate ions from MTA cement with CDHP and the ability of calcium phosphate deposition should, therefore, be investigated in the future.

Based on these results, it is considered that the addition of 0.4 g/mL of CDHP to MTA cement enhances low crystalline hydroxyapatite precipitation, which in turn possibility enhances the ability to form restorative dentin.

CONCLUSION

The addition of an appropriate amount of CDHP to MTA cement is useful for improving cytotoxicity in the early stages of the cement setting, and also contributes to a shorter setting time and higher strength relative to the system without CDHP. Furthermore, this study showed that the addition of 0.4 g/mL CDHP to MTA cement was effective in enhancing deposition of low crystalline hydroxyapatite on the MTA cement without reducing its physical properties.

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