In vitro and *in vivo* characteristics of fluorapatite-forming calcium phosphate cement with calcium silicate for endodontic applications

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The following two articles are part of this doctoral dissertation:
    Yusuke Suzuki et al. *Dental Materials Journal*, 2015 *(in press)*
    Yusuke Suzuki et al. *Journal of Oral Science*, 2015 *(in press)*

Abstract

Ideal orthograde and/or retrograde filling materials in endodontic treatments should seal off the pathways of communication between the root canal system and its surrounding periodontal tissues. Additionally, these materials should be biocompatible with the host tissues, insoluble in tissue fluids, and easily manipulated. Mineral trioxide aggregate (MTA) is a promising biomaterial that is widely used in various endodontic treatments due to its excellent biocompatibility, superior sealing, and its ability to set in the presence of blood. However, a disadvantage of this material is its difficult handling properties, namely, relatively long setting times. Thus, a need has been identified for the development of materials more suitable for endodontic treatments.

Calcium phosphate cement (CPC) that initially contains an equimolar mixture of tetracalcium phosphate (TTCP), $Ca_4(PO_4)_2O$ and dicalcium phosphate anhydrous (DCPA), $CaHPO_4$ has self-hardening characteristics and forms hydroxyapatite (HA) as the end product. The CPC has become a subject of interest in dental and medical applications as bone graft biomaterials, due to its setting property, biocompatibility, and osteoconductivity. It is important to note that CPC of different compositions can form different end products in addition to HA, such as fluorapatite (FA). Because the end

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products of CPC may control resorption rates, CPC can be developed to have resorption rates suitable for diverse clinical applications.

For endodontic treatments such as root-end filling, perforation repair, apexification, pulp-capping, or pulpotomy, it is desirable to have CPC that is biocompatible and osteoconductive, yet non-bioresorbable in soft and hard tissue. Because dissolution in a cell-mediated acidic environment leads to *in vivo* resorption, CPC that forms end products that have little or practically no solubility in such acidic conditions can be predicted to be essentially non-resorbable. It is well described in the literature that FA has significantly lower solubility in acids and also promotes bone formation in the rat tibia and dog mandible. In addition, it has been previously described that the addition of tricalcium silicate (TCS) improved the sealing ability and raised the pH of CPC to levels comparable to that produced by MTA. Hence, FA-forming CPC with TCS can be expected to have much lower resorbability than HA-forming CPC, good sealing ability, and sufficient alkalinity.

The purpose of this study was to develop a novel FA-forming CPC with TCS for endodontic applications and to examine its physiochemical properties and biocompatibilities.

In chapter 1, physiochemical properties of FA-forming CPC with TCS were

examined *in vitro*, and its biocompatibility with rat subcutaneous connective tissue was also assessed *in vivo*. The FA-forming CPC powder consisted of 62.8% $CaHPO_4$, 30.8% $CaCO_3$, and 6.4% NaF. One part of TCS was combined with 9 parts of FA-forming CPC powder (FA-forming CPC with TCS). A 1.5 M phosphate solution was used as cement liquid. Setting time (ST), diametral tensile strength (DTS), phase composition by X-ray diffraction (XRD), and cement alkalinity were analyzed. Cement biocompatibility was assessed using rat subcutaneous model. Cement ST was 10.3±0.6 min and DTS was 3.89±0.76 MPa. XRD patterns showed that highly crystalline apatitic material was the only significant phase present and pH value was approximate 11.0. FA-forming CPC with TCS demonstrated similar biocompatibility as that of MTA control. These results of chapter 1 showed that FA-forming CPC with TCS has appropriate characteristics as endodontic cement in not only physicochemical but also biocompatible analyses.

In chapter 2, the effect of FA-forming CPC with TCS on cultured osteoblast-like cells (ROS 17/2.8 cells; ROS cells) was investigated *in vitro*. Cell culture was performed using cell culture insert method whereby each test material was placed on porous membrane insert in cell culture plate. Proliferation, morphological change, and alkaline phosphatase (ALP) activity in ROS cells were measured in the presence of FA-forming CPC with TCS, compared with MTA. A logarithmic growth phase and

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cellular morphological changes were identical in ROS cells in all experimental groups. Additionally, no significant ALP activity in ROS cells was noted between treatment with FA-forming CPC with TCS and MTA. The present results indicate that FA-forming CPC with TCS has characteristics identical to those of MTA under this experimental condition.

Within the limitation of these studies, the results suggested that FA-forming CPC with TCS has an appropriate characteristic as endodontic cements in the both *in vitro* and *in vivo* analyses. Therefore, FA-forming CPC with TCS may be useful for endodontic applications.

Chapter 1: Development of a novel fluorapatite-forming calcium phosphate cement with calcium silicate: *In vitro* and *in vivo* characteristics

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Introduction

Major endodontic failures occur as a result of leakage of irritants from pathologically involved root canals into the periradicular tissues (1). Ideal orthograde and/or retrograde filling materials should seal off the pathways of communication between the root canal system and its surrounding periodontal tissues (2). Additionally, these materials should be biocompatible with the host tissues, insoluble in tissue fluids, and easily manipulated (3). Although many types of endodontic materials, such as gutta-percha, zinc oxide eugenol-based cement, composite resin, and glass ionomer cement, have been introduced over the years, most exhibit different levels of weakness. Mineral trioxide aggregate (MTA) materials, developed in 1993 by Torabinejad *et al*. (3), overcame most of these weaknesses and have been in widespread use of endodontic treatments. However, an undesirable property of this material was its difficult handling

properties, namely, relatively long setting times (4). Thus, it is needed to develop even more ideal materials for endodontic treatments.

Calcium phosphate cement (CPC) that initially contains an equimolar mixture of tetracalcium phosphate (TTCP), $Ca_4(PO_4)_2O$ and dicalcium phosphate anhydrous (DCPA), $CaHPO_4$ has self-hardening characteristics and forms hydroxyapatite (HA) as the end product (5). The CPC has become a subject of interest in dental and medical applications as bone graft biomaterials, due to its setting property, biocompatibility, and osteoconductivity (6, 7). More importantly, it is gradually replaced by new bone without volume loss (7). It is important to note that CPC of different compositions can form different end products in addition to HA, such as dicalcium phosphate dehydrate (8), octacalcium phosphate (9), or fluorapatite (FA) (10). Because the end products of CPC may control resorption rates, CPC can be developed to have resorption rates suitable for diverse clinical applications.

Takagi *et al.* (11) developed self-hardening CPCs based on DCPA and CaO or $CaCO_3$. A preliminary study indicated that the addition of sodium fluoride (NaF) to these CPCs also formed fluoridated HA or FA and CaF_2 (12).

For endodontic treatments such as root-end filling, perforation repair, apexification, pulp-capping, or pulpotomy, it is desirable to have CPC that is biocompatible and

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osteoconductive, yet non-bioresorbable in soft and hard tissue. Because dissolution in a cell-mediated acidic environment leads to *in vivo* resorption, CPC that forms end products that have little or practically no solubility in such acidic conditions can be predicted to be essentially non-resorbable. It is well described in the literature that fully or partially fluoridated HA materials have significantly lower solubility in acids (13) and also promote bone formation in the rat tibia and dog mandible (14, 15). Additionally, Cherng *et al.* (16) showed that the addition of tricalcium silicate (TCS) improved the sealing ability and raised the pH of CPC to levels comparable to that produced by MTA. Hence, FA-forming CPC with TCS can be expected to have much lower resorbability than HA-forming CPC, good sealing ability, and sufficient alkalinity. The purpose of this study was to develop a novel FA-forming CPC with TCS for endodontic applications and to examine its physiochemical properties and biocompatibility.

Materials and Methods

Test materials

All chemicals used in this study were reagent grade purchased from J.T. Baker Chemical Co. (NJ, USA). FA-forming CPC powder without TCS consisted of 62.8% (% denotes mass percent) DCPA ($CaHPO_4$), 30.8% calcium carbonate ($CaCO_3$), and 6.4%

sodium fluoride (NaF), as described previously (17). A batch of DCPA with a median

particle size of 6.5±0.3 μm (n=3) was produced by grinding DCPA in a blender (model

38BL52 LBC10, Waring Commercial, Torrington, CT, USA). $CaCO_3$ was ground in a

planetary ball mill (Retsch PM4, Brinkman, NY, USA) in cyclohexane for 24 h to

produce a median particle size of 2.6±0.4 μm (n=3). TCS was prepared by combining 3

moles of CaO with 1 mole of fumed silica (SiO_2) in water and stirred for 8 h. The slurry

was filtered, dried, and then heated at 1450°C. The resulting powder was ground to a

median size of 20 μm. To prepare FA-forming CPC powder with TCS, one part of TCS

was added to 9 parts of FA-forming CPC powder. A 1.5 M sodium phosphate solution

(pH 5.6) was used as the cement liquid. MTA (ProRoot MTA, Dentsply Tulsa, TN,

USA) was used according to manufacturers' instructions as the control.

Physicochemical analysis

1. Setting time (ST) and diametral tensile strength (DTS) measurements

All samples for ST and DTS measurements were prepared by mixing cement

powder and liquid at the powder (g)/liquid (mL) ratio (P/L) of 3 to produce a cohesive

paste. For the ST measurement, the paste was packed into a stainless steel mold

(diameter: 6 mm; height: 3 mm) sandwiched between two glass plates, and stored in a

humidor with 100% relative humidity at 37°C. The ST was measured using the

Gillmore needle method (ADA specification #9). The test was performed using a needle with a tip diameter of 1.06 mm loaded with 453.5 g of weight. In this method, the cement was considered to have set when the needle with the load failed to make a perceptible indentation on the surface. The ST was the average time obtained from 3 specimens.

For the DTS measurement, specimens were prepared as described previously (18). The paste was packed into a stainless-steel mold, formed by two rods (diameter: 6 mm) in a cylindrical cavity, with 2.8 MPa of applied pressure; the mold was placed in an incubator at 37°C and 100% humidity for 4 h. The specimens were then removed from the molds, and individually immersed in 10 mL of a physiological-like solution (PLS) (19), containing 1.15 mM Ca, 1.2 mM P, 133 mM NaCl, and 50 mM HEPES buffer, with the pH adjusted to 7.4, at 37°C for 20 h. After immersion in PLS, the diameter and length of each specimen were measured with a micrometer, and the sample was placed on a universal testing machine (United Calibration Corp, Garden Grove, CA, USA) for the DTS measurement. The specimen was stressed between steel platens that were covered with one thickness of wet filter paper and crushed at a loading rate of 10 mm/min. The force at failure was recorded and converted into a stress in MPa unit. The DTS value presents the average value obtained from 5 specimens.

2. X-ray diffraction (XRD) and cement alkalinity analyses

The phases present in the FA-forming CPC with TCS were determined by powder

XRD analysis. After the DTS measurement, the fractured specimens were dehydrated in

100% ethanol for 1 h and dried in a desiccator for 3 days. The dried specimens were

ground into a fine powder with a mortar and pestle, and characterized by XRD. The

XRD patterns of the specimens were recorded using a vertically-mounted diffractometer

system (D/MAX 2000, Rigaku, Danvers, MA, USA), with graphite-monochromatized

CuK_α radiation (λ=0.1540 nm) generated at 40 kV and 40 mA. The sample was scanned

from 10 to 50 degrees 2θ in continuous mode ($2° \ 2\theta \ min^{-1}$, time constant 2 sec). The

unreacted powder of FA-forming CPC with TCS was also assessed using the same XRD

procedure.

The alkalinity of FA-forming CPC with or without TCS, and that of MTA was

measured using a pH meter (pH 500 series, Cole-Parmer, Vernon Hills, IL, USA). The

pH values of a 1 mL aliquot of 30 mM KCl solution after the addition of 0.25 g of

pulverized set FA-forming CPC with or without TCS or set MTA were measured for 10

min to evaluate cement alkalinity. The set sample was ground by a mortal and pestle,

and 0.25 g of the sample was placed in 1 mL of 30 mM KCl solution. A flat-end

combination pH electrode (Thomas Scientific, model #S450CBNC, Swedesboro, NJ,

USA) was connected to the pH meter, and the pH was measured continuously for 10 min. The sample suspension was under constant stirring (350 rpm) and the pH of the solution was monitored. The standard uncertainty of the pH measurement was estimated to be 0.01 pH units.

Biocompatibility analysis

The biocompatibility of FA-forming CPC with TCS in the subcutaneous tissue of rats was examined. This study was permitted by and performed under guidelines specified by the Animal Experimentation Committee at Nihon University School of Dentistry, with experiments carried out at the Nihon University School of Dentistry animal laboratory.

1. Animal preparation and surgical procedure

Thirty seven-week-old male Donryu rats, weighing 200–250 g, were divided into three test periods (7, 21, or 42 days; n=10 in each experimental period). Material implantation was performed using procedures modified from a previously method (10). Briefly, each animal was anesthetized by intraperitoneal injection of pentobarbital sodium (Kyoritsu Seiyaku Co., Tokyo, Japan) at a dose of 50 mg/kg body weight. Under general anesthesia, the back area of the rat was shaved and swabbed with 70% volume fraction ethanol. Subcutaneous pockets were created to receive test materials in the

dorsal region of the rat. Horizontal incisions approximately 15 mm in length were made along each side of the back bone, and subcutaneous skin pockets were created by blunt dissection. To prevent interactions among the materials being tested, the tubes were placed at least 20 mm apart.

FA-forming CPC with TCS was prepared for biocompatibility analysis according to a previously described method (20), meanwhile, MTA was prepared according to the manufacturers' instructions. Sterile polyethylene tubes (inner diameter: 0.8 mm; length: 8 mm) with one end closed were divided into 3 groups: negative control group (empty polyethylene tubes), an MTA group (polyethylene tube filled with MTA), and an FA-forming CPC with TCS group (polyethylene tube filled with FA-forming CPC with TCS). After mixing of these cements, these were immediately filled into the tubes. Each of these tubes was inserted into a pocket of subcutaneous tissues, and then the pocket was closed with interrupted sutures.

2. Tissue preparation and histological evaluation

Each group of animals was euthanized by anesthesia overdose at the end of each time period. The implants and surrounding tissues were carefully removed and fixed in 10% neutral formalin for 2 weeks, and the specimens were embedded in paraffin. Tissue sections were made longitudinally through the middle of the tubes and 3 sections from

each specimen were selected randomly. These sections were subjected to hematoxylin and eosin (H-E) staining for histopathological evaluation.

Histopathological evaluation was performed in microscopic fields adjacent to experimental materials at the open ends of the tubes under a light microscope (DFC500, Leica Microsystems, Wetzlar, Germany) at ×100, ×200, and ×400 magnifications by an observer that was blind to the procedure. Evaluation of the inflammatory reaction was conducted according to Cox and Robbin's criteria (21, 22) focused on the accumulation of acute and chronic inflammatory cells, fibrin deposits, tissue edema, and vascular congestion. These criteria were classified into 4 grades: Grade I, scattered and wavy collagen fiber deposits and fibrosis; Grade II, infiltration of inflammatory cells, and wavy collagen fiber deposits and fibrosis; Grade III, dense infiltration of inflammatory cells, limited areas of tissue edema and vascular congestion; and Grade IV, very dense infiltration of acute and chronic inflammatory cells, widespread edematous areas and vascular congestion along with fibrin deposits.

Statistical analysis

Statistical analysis on ST and DTS values of FA-forming CPC with TCS and FA-forming CPC without TCS was performed using Student's *t*-test. The significance of the differences in the severity of inflammation was determined using two-way ANOVA

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and Tukey's test. Statistical significance was defined as $p<0.05$.

Results

Physicochemical analysis

1. ST and DTS measurements

The ST (mean±SD: n=3) of FA-forming CPC with and without TCS was 10.3±0.6 min and 14.0±1.0 min, respectively. The DTS (mean±SD: n=5) of FA-forming CPC with and without TCS was 3.89±0.76 MPa and 2.65±0.29 MPa, respectively (Table 1). ST decreased with the addition of TCS while, in contrast, DTS increased with the addition of TCS. A significant difference was detected between these cements in both properties.

2. XRD and cement alkalinity analyses

The powder XRD pattern of FA-forming CPC with TCS showed that highly crystalline apatitic material was the main phase in the hardened cement (Fig. 1). The pH values of FA-forming CPC with and without TCS, and that of MTA all increased rapidly after the addition of 0.25 g of pulverized set cements and became nearly constant at approximately 9.0, 11.0 and 13.0, respectively (Fig. 2).

Biocompatibility analysis

1. Period of 7 days (Fig.3 a, b, and c)

The inflammatory response among all groups was similar in period of 7 days. The main characteristics are the presence of infiltration of inflammatory cells with collagen fiber deposits and limited areas of vascular congestion. In the FA-forming CPC with TCS group, 3.3, 46.7, and 50.0% of the specimens indicated Grade I, Grade II, and Grade III inflammation, respectively. In the MTA group, 3.3, 40.0, and 56.7% of the specimens had Grade I, Grade II, and Grade III inflammation, respectively. In the control group, 6.7, 60.0, and 33.3% of the specimen revealed Grade I, Grade II, and Grade III inflammation, respectively.

2. Period of 21 days

The inflammatory response for all groups decreased with time. A thick fibrous capsule formation around open-end of the polyethylene tube was observed. In the FA-forming CPC with TCS group, 66.7, 23.3, and 10.0% of the specimens demonstrated Grade I, Grade II, and Grade III inflammation, respectively. In the MTA group, 33.3, 63.3, and 3.3% of the specimen exhibited Grade I, Grade II, and Grade III inflammation, respectively. In the control group, 60.0, 36.7, and 3.3% of the specimens indicated Grade I, Grade II, and Grade III inflammation, respectively.

3. Period of 42 days (Fig. 3 d, e, and f)

The tissue response of three groups was very similar to the 21 days period. In the

FA-forming CPC with TCS group, 86.7 and 13.3% of the specimens indicated Grade I

and Grade II inflammation, respectively. In the MTA group, 83.3 and 16.7% of the

specimens had Grade I and Grade II inflammation, respectively. In the control group,

90.0 and 10.0% of the specimens revealed Grade I and Grade II inflammation,

respectively.

4. Comparison of inflammatory response

Means of inflammation grades in histopathological evaluations of all experimental

groups are indicated in Fig. 4. Within the same experimental period, no-significant

difference was detected among the groups. In contrast, the mean inflammation grade for

the period of 7 days was significantly higher than those for 21 days and 42 days in the

same group ($p<0.01$). No-significant difference was observed between 21 and 42 days

for control and FA-forming with TCS groups ($p>0.05$), however, the MTA group

exhibited a significant difference ($p<0.01$).

Discussion

MTA has been used widely in endodontic treatments, due to not only its superior

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sealing ability but also its biocompatibility. Nevertheless, one of the main drawbacks of

MTA is its extended setting period and prolonged maturation phase. The longer setting

time of MTA, in comparison with Portland cement, is attributed to the lower levels of

sulfur and tricalcium aluminate in MTA (23). Although many have attempted to shorten

the setting time of MTA by various accelerators to overcome this clinical disadvantage

(24, 25), adding various elements can adversely affect the ideal characteristics of MTA

(4). Therefore, FA-forming CPC with TCS was developed as a novel endodontic

cement. The ST of FA-forming CPC with TCS (10.3±0.6 min) was significantly shorter

than that of the cement without TCS (14±1.0 min). Additionally, the ST of FA-forming

CPC with TCS is much shorter than the initial setting time of MTA using the Gillmore

needle method according to ASTM C266, which was reported to 45±2.9 min (26).

When used for various endodontic treatments, this difference may be of important

clinical significance.

The DTS value of FA-forming CPC with TCS was significantly higher than that

without TCS as determined in the present study. Huang *et al*. (26) reported a DTS value

of 4.4±0.1 MPa for a 24-h specimen of MTA, which is marginally higher than the DTS

(3.89±0.76 MPa) of FA-Forming CPC with TCS. Endodontic materials do not bear

direct pressure during function, hence, a high DTS value is not believed to be as an

important requirement as for materials used to repair or restore defects in load-bearing sites. Thus, FA-forming CPC with TCS should be sufficient for most endodontic applications and was focused in the powder XRD pattern analysis.

The powder of FA-forming CPC with TCS used in this study contained NaF as a source of fluoride. When mixed with phosphate solution, the powder XRD pattern (Fig. 1) revealed that highly crystalline apatitic material was the only significant phase present. It is known that FA typically has very high crystallinity compared to HA, and also TCS was added to the FA-forming CPC (12), final product of the FA-forming CPC with TCS was FA. FA has the more stable characteristic than HA because FA is significantly less soluble than HA or tooth mineral itself under neutral and acidic pH conditions (13). Therefore, FA-forming CPC with TCS may be suitable for endodontic applications that require the material to have high stability for a long time. Although Takagi *et al*. (10) described that a CPC that contains TTCP and DCPA mixed with an H_3PO_4 solution including hydrogen fluoride could also form FA, the cement that uses NaF as a source of fluoride in this study may be clinically desirable from a safety viewpoint, because of the hazardous nature of hydrogen fluoride.

Present results showed that higher alkalinity of FA-forming CPC with TCS compared to that without TCS. This was attributed to the highly alkaline TCS

compound. The pH value of MTA was higher than that of FA-forming CPC with TCS.

Calcium hydroxide, an important endodontic materials, is generally effective at

eradicating intraradicular bacteria due to its alkaline pH (27). Tronstad *et al*. (28)

demonstrated that the pH values of dentin adjacent to calcium hydroxide change their

pH range to 11.0–12.2 after calcium hydroxide is placed in the root canals. Because the

pH value of FA-forming CPC with TCS is similar to that of calcium hydroxide,

FA-forming CPC with TCS may maintain the local state of alkalinity necessary for bone

or dentin formation.

Because the characteristics of ST, DTS, and cement alkalinity for endodontic

applications of FA-forming CPC with TCS is better than those of FA-forming CPC

without TCS, the biocompatibility analyses of FA-forming CPC with TCS and MTA

were analyzed. These analyses used in this study involved subcutaneous implant

methods, in which the materials to be evaluated were placed in polyethylene tubes that

were implanted in the subcutaneous connective tissue in rats. This method was

introduced by Torneck (29) and has become one of the most common methods for the

evaluation of dental-material biocompatibility. The placement of the material to be

evaluated in the terminal portions of the polyethylene tubes prevented the diffusion of

the material into the connective tissue; thus, this method is preferred to direct injection

into the subcutaneous connective tissue (30). Several inflammatory assessment methods of tissue response adjacent to materials have been used in rat connective tissue. This study used Cox and Robin's criterion, which is based on the accumulation of acute and chronic inflammatory cells, fibrin deposits, tissue edema, and vascular congestion. This innovative method for assessment is considered to be more precise for comparison of the severity of the inflammation around the tubes containing the materials, due to the attention to vascular and reparative response (31).

In the biocompatibility analysis, the inflammatory response decreased over time in all three groups. The mean inflammation grade in each group at 7 days was significantly higher than those at 21 and 42 days. At the same time no-significant differences were observed among the three groups for each of the three time periods. This demonstrates that neither FA-forming CPC with TCS nor MTA stimulated the surrounding tissues more than the negative control group (empty tube). After 21 days, the mean inflammation grade decreased significantly in each group, with no-significant difference among the three groups. Additionally, no-significant difference was evident between 21 and 42 days for the control and FA-forming CPC with TCS groups; however, the MTA group exhibited a significant difference. Consequently, the severity of the inflammatory responses adjacent to FA-forming CPC with TCS and MTA were identical, and equal to

that of the negative control group during this experimental period. Thus, it is thought

that FA-forming CPC with TCS presented the same tissue response and biocompatibility

as MTA, which is known for its excellent biocompatibility, when tested in rat

subcutaneous connective tissue.

According to the findings of chapter 1, it is concluded that the setting time of

FA-forming CPC with TCS is shorter than that of MTA and it can form FA and presents

adequate DTS, pH value, and biocompatibility as endodontic cements. Therefore,

FA-forming CPC with TCS may be useful for endodontic applications.

Chapter 2: Effect of a novel fluorapatite-forming calcium phosphate cement with calcium silicate on osteoblasts in comparison with mineral trioxide aggregate

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Introduction

The results of chapter 1 indicated that FA-forming CPC with TCS has an appropriate physiochemical characteristic and biocompatibility with rat subcutaneous connective tissue as endodontic cements. However, the effect of FA-forming CPC with TCS on the proliferation and alkaline phosphatase (ALP) activity of osteoblasts has not been determined. Therefore, proliferation, morphologic changes, and ALP activity induced by the by the novel endodontic cement FA-forming CPC with TCS and by MTA were evaluated and compared in the present study.

Materials and Methods

Test materials

The same test materials as those in chapter 1 were used in this study. Each test material (diameter: 3 mm; thickness: 0.5 mm) was allowed to set for 24 h at 37°C and

100% humidity, and placed in α-minimal essential medium (α-MEM; Gibco BRL, Rockville, MD, USA) (0.7 mL) for 3 days as described previously by Takita *et al*. (32).

Cell culture

The rat clonal osteoblast line ROS 17/2.8 (ROS cells) was used in this study. The cells were maintained in growth medium consisting of α-MEM supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Logan, UT, USA) and 1% (vol/vol) penicillin–streptomycin-neomycin solution (PSN; Sigma Chemical, St. Louis, MO, USA) under standard culture conditions (37°C, 100% humidity, 95% air, and 5% CO_2).

Cell proliferation and morphology

Cell proliferation in the presence of the test materials was measured using 24-well cell culture plates. Each well contained a culture plate insert with a porous membrane (3-µm pore size, BD Falcon, Franklin Lakes, NJ, USA) as described previously by Ogata *et al*. (33). Briefly, ROS cells were plated at a density of 2.0×10^4 cells per well in 500-µL α-MEM containing 10% (vol/vol) FBS. After incubation for 24 h, a culture plate insert with one pellet of the test material was put into each well (Fig. 5). Cells cultured without the test material served as the control. Cells were enumerated using a Cell Counting Kit-8 (Dojindo Molecular Technologies Inc., Kumamoto, Japan) after 3,

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5, 7, and 9 days, at which time points the medium was replaced with fresh medium containing 10% (vol/vol) cell-counting reagent after observation of changes in cell morphology by light microscopy (DIAPHOT, Nikon, Tokyo, Japan). After further incubation for 1 h, a microtiter plate reader (Titertec Multiskan Plus, Flow Laboratory, McLean, VA, USA) was used to measure intensity of the reaction products at 450 nm. The number of cells was calculated from the absorbance value on the basis of a standard curve.

ALP activity

ALP activity in the presence of the test materials was measured using previously described methods (33). Briefly, ROS cells were seeded onto 24-well microplates at a density of 2×10^4 /well for up to 9 days using cell culture inserts, as described above (Fig. 5). At each timepoint, 500 µL of enzyme assay solution (8 mM p-nitrophenyl phosphate, 12 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.1 M glycine-NaOH buffer; pH 10.5) were added to the cells in each well, and the plate was incubated for several min at 37°C. The enzymatic reaction was terminated by the addition of 500 µL of 0.1 M NaOH. The amount of p-nitrophenol released by the enzymatic reaction was determined by measuring absorbance at 405 nm using a microtiter plate reader. ALP activity was defined as the amount of p-nitrophenol produced per minutes.

Statistical analysis

The results of ALP activity were statistically analyzed using one-way analysis of variance and the Bonferroni test of an average of three replicates. A *p* value of <0.05 was considered to indicate statistical significance. Data are expressed as mean±standard deviation.

Results

Cell proliferation and cell morphology

Proliferation of ROS cells was determined in the presence of FA-forming CPC with TCS, MTA, or control for up to 9 days of culture (Fig. 6). The logarithmic growth phase was equal in all cultures at 3 and 5 days after seeding. Light micrographs of ROS cells were obtained at each timepoint. In all experimental groups, ROS cells grew and formed a confluent monolayer up to day 9 of culture. There were no morphologic differences between the test materials and control at any time point (Fig. 7).

ALP activity

ALP activity was determined in ROS cells up to day 9 of culture. The only significant difference observed was between the control and MTA groups at 3 days of culture (Fig. 8).

Discussion

To minimize incidences of local and systemic adverse effects, the biocompatibility of endodontic materials should be investigated in *in vitro* and *in vivo* tests before their clinical application (34). Many parameters can be used to characterize the biocompatibility of an endodontic material, including genotoxicity, carcinogenicity, cytotoxicity, histocompatibility and antimicrobial effects (34). Numerous cell culture studies have evaluated cytotoxic reactions induced by endodontic materials. These studies focused on growth inhibition, synthesis of DNA, RNA or protein, and alternations in cell morphology (35-39). In this study, responses of osteoblast-like cells to FA-forming CPC with TCS were evaluated and compared with MTA in relation to proliferation, morphologic alterations, and ALPase activity.

Proliferation of ROS cells in the presence of FA-forming CPC with TCS was similar to that in the presence of the control or MTA. A previous study found that MTA was one of the least cytotoxic dental materials (40). The present results indicate that FA-forming CPC with TCS had excellent biocompatibility, similar to that of MTA. Recently, it has been reported that FA-forming CPC with TCS and MTA are highly biocompatible with rat subcutaneous connective tissue as described in chapter 1. The proliferation of ROS cells observed in the present study is consistent with this finding. In aqueous conditions,

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FA-forming CPC with TCS is almost entirely converted to FA within 24 h as described

in chapter 1. FA typically has very high crystallinity, as compared with HA, and good

biocompatibility with not only osteoblasts but also dental pulp cells, mesenchymal cells,

and endothelial cells (41, 42). The excellent biocompatibility of FA-forming CPC plus

TCS with ROS cells is attributable to its conversion to FA.

Furthermore, FA-forming CPC with TCS, MTA, and the control had equally

favorable biocompatibility and no effect on cell morphology. Biocompatibility with cell

morphology may also be due to the conversion to biocompatible FA. Haglund *et al.* (43)

reported that MTA caused lysis of adjacent cells and denaturation of medium proteins

because, when freshly mixed, MTA releases various cytotoxic chemical by-products.

However, when set, MTA is biocompatible with cells. Therefore, all test materials were

allowed to set for 24 h in 100% humidity and then put in α-MEM for 3 days before cell

culture, for the assessment of cell morphology in set test materials.

ALP hydrolyzes the ester bond of organic phosphate compounds under alkaline

conditions and is important in bone calcification (44). In this study, ALP activity was

used to assess cell differentiation in test materials. The only significant difference in

ALP activity was between the control and MTA groups at 3 days of culture. Lui *et al.*

(45) reported that FA showed good biocompatibility with osteoblast-like MG-63 cells,

and reported long-term growth and differentiation of these cells. Because FA-forming

CPC with TCS is converted to biocompatible FA, ALP activities in ROS cells are in

agreement with their results (45). The statistically significant difference between the

control and the MTA groups at 3 days of culture is likely due to the small amount of

residual chemical by-products of MTA, which may be present for up to 3 days of culture,

even if MTA is adequately immersed in α-MEM before cell culture.

On the basis of present findings of chapter 2, FA-forming CPC with TCS and

MTA—the gold standard endodontic cement—had similar effects on proliferation,

morphology, and ALP activity in ROS cells.

Conclusions

The purpose of this study was to investigate the *in vitro* and *in vivo* characteristics of FA-forming CPC with TCS. From the results of chapter 1 and 2, the following conclusions were drawn:

1. ST and DTS of FA-forming CPC with TCS are shorter and higher than those of FA-forming CPC without TCS, respectively.

2. The pH value of FA-forming CPC with TCS indicates approximately 11.0, between those of FA-forming CPC without TCS and MTA.

3. FA-forming CPC with TCS has similar biocompatible characteristics with rat subcutaneous connective tissue and osteoblasts in comparison with MTA.

Within the limitation of the present *in vitro* and *in vivo* studies, FA-forming CPC with TCS may be useful for endodontic applications.

Acknowledgements

References

1. Siqueira JF Jr, Rocas IN (2008) Clinical implications and microbiology of bacterial persistence after treatment procedures. J Endod 34, 1291-1301.

2. Chng HK, Islam I, Yap AU, Tong YW, Koh ET (2005) Properties of a new root-end filling material. J Endod 31, 665-668.

3. Torabinejad M, Watson TF, Pitt Ford TR (1993) Sealing ability of a mineral trioxide aggregate when used as a root end filling material. J Endod 19, 591-595.

4. Parirokh M, Torabinejad M (2010) Mineral trioxide aggregate: A comprehensive literature review-Part III: Clinical applications, drawbacks, and mechanism of action. J Endod 36, 400-413.

5. Brown WE, Chow LC (1986) A new calcium phosphate, water-setting cement. In: Cement research progress 1986, Brown PW ed, American ceramic society, Westerville, 352-379.

6. Sugawara A, Nishiyama M, Kusama K, Moro I, Nishimura S, Kudo I et al. (1992) Histopathological reactions of calcium phosphate cement. Dent Mater J 11, 11-16.

7. Sugawara A, Fujikawa K, Takagi S, Chow LC (2008) Histological analysis of calcium phosphate bone grafts for surgically created periodontal bone defects in dogs. Dent Mater J 27, 787-794.

8. Mirtchi AA, Lemaitre J, Terao N (1989) Calcium phosphate cements: study of the β-tricalcium phosphate-monocalcium phosphate system. Biomaterials 10, 475-480.

9. Markovic M, Chow LC (2010). An octacalcium phosphate forming cement. J Res Natl Inst Stand Technol 115, 257-265.

10. Takagi S, Frukhtbeyn S, Chow LC, Sugawara A, Fujikawa K, Ogata H et al. (2010) In vitro and in vivo characteristics of fluorapatite-forming calcium phosphate cements. J Res Natl Inst Stand Technol 115, 267-276.

11. Takagi S, Chow LC, Ishikawa K (1998) Formation of hydroxyapatite in new calcium phosphate cements. Biomaterials 19, 1593-1599.

12. Takagi S, Ogata H, Chow LC (2011) Properties of DCPA+CaO and DCPA+CaCO$_3$ fluorapatite-forming calcium phosphate cements. J Dent Res 90, Spec Iss B 2476. (Abstract)

13. Chow LC, Markovic M (1998) Physicochemical properties of fluorapatite. In: Calcium phosphates in biological and industrial systems, Amjad Z ed, Kluwer Academic, Norwell, 67-84.

14. Sakae T, Ookubo A, LeGeros RZ, Shimogoryou R, Sato Y, Lin S et al. (2003) Bone

formation induced by several carbonate and fluoride containing apatite implanted in dog mandible. Key Eng Mater 240, 395-398.

15. Inoue M, Rodriguez AP, Nagai N, Nagatsuka H, LeGeros RZ, Tsujigiwa H et al. (2011) Effect of fluoride-substituted apatite on *in vivo* bone formation. J Biomater Appl 25, 811-824.

16. Cherng AM, Takagi S, Chow LC (2010) Acid neutralization capacity of a tricalcium silicate-containing phosphate cement as an endodontic materials. J Res Natl Inst Stand Technol 115, 471-476.

17. Chow LC, Takagi S, Wang W, Frukhtbeyen S (2013) A new fluorapatite and calcium silicate cement for endodontic treatments. J Dent Res 92, Spec Iss A 487. (Abstract)

18. Chow LC, Hirayama S, Takagi S, Parry E (2000) Diametral tensile strength and compressive strength of a calcium phosphate cement: Effect of applied pressure. J Biomed Mater Res 53, 511-517.

19. Takagi S, Chow LC, Hirayama S, Sugawara A (2003) Premixed calcium-phosphate cement pastes. J Biomed Mater Res B Appl Biomater 67, 689-696.

20. Suzuki Y, Hayashi M, Ogata H, Yasukawa T, Takagi S, Chow LC et al. (2013) Biocompatibility of fluorapatite cement with calcium silicate for endodontic treatment. J Dent Res 92, Spec Iss B 123. (Abstract)

21. Robbins S, Cortan R, Kumar V (1991) Pocket compaction to Robbins pathologic basis of diseases. 6th ed, WB Saunders, Philadelphia, 45-46.

22. Cox CF, Subay RK, Suzuki S, Suzuki SH, Ostro E (1996) Biocompatibility of various dental materials: pulp healing with a surface seal. Int J Periodontics Restorative Dent 16, 240-251.

23. Dammaschke T, Gerth HU, Zuchner H, Schafer E (2005) Chemical and physical surface and bulk material characterization of white ProRoot MTA and two Portland cements. Dent Mater 21, 731-738.

24. Kogan P, He J, Glickman GN, Watanabe I (2006) The effects of various additives on setting properties of MTA. J Endod 32, 569-572.

25. Wiltbank KB, Schwartz SA, Schindler WG (2007) Effect of selected accelerants on the physical properties of mineral trioxide aggregate and Portland cement. J Endod 33, 1235-1238.

26. Huang TH, Shie MY, Kao CT, Ding SJ (2008) The effect of setting accelerator on properties of mineral trioxide aggregate. J Endod 34, 590-593.

27. Pawinska M, Skrzydlewska E (2003) Release of hydroxyl ions from calcium

hydroxide preparations used in endodontic treatment. Rocz Akad Med Bialymst 48, 145-149.

28. Tronstad L, Andreasen JO, Hasselgren G, Kristerson L, Riis I (1981) pH changes in dental tissues after root canal filling with calcium hydroxide. J Endod 7, 17-21.

29. Torneck CD (1966) Reaction of rat connective tissue to polyethylene tube implants-Part I. Oral Surg Oral Med Oral Pathol 21, 379-387.

30. Olsson B, Sliwkowski A, Langeland K (1981) Subcutaneous implantation for the biological evaluation of endodontic materials. J Endod 7, 355-367.

31. Shahi S, Rahimi S, Lotfi M, Yavari H, Gaderian A (2006) A comparative study of the biocompatibility of three root-end filling materials in rat connective tissue. J Endod 32, 776-780.

32. Takita T, Hayashi M, Takeichi O, Ogiso B, Suzuki N, Otsuka K et al. (2006) Effect of mineral trioxide aggregate on proliferation of cultured human dental pulp cells. Int Endod J 39, 415-422.

33. Ogata H, Hayashi M, Tsuda H, Suzuki N, Maeno M, Sugawara A et al. (2012) Effects of a calcium phosphate cement on mineralized nodule formation compared with endodontic cements. Dent Mater J 31, 92-97.

34. Geurtsen W, Leyhausen G (1997) Biological aspects of root canal filling materials-histocompatibility, cytotoxicity, and mutagenicity. Clin Oral Investig 1, 5-11.

35. Matsumoto K, Inoue K, Matsumoto A (1989) The effect of newly developed root canal sealers on rat dental pulp cells in primary culture. J Endod 15, 60-67.

36. Al-Nazhan S, Spangberg L (1990) Morphological cell changes due to chemical toxicity of a dental material: An electron microscopic study on human periodontal ligament fibroblasts and L929 cells. J Endod 16, 129-134.

37. McNamara JR, Heithersay GS, Wiebkin OW (1992) Cell responses to Hydron by a new *in-vitro* method. Int Endod J 25, 205-212.

38. Barbosa SV, Burkard DH, Spangberg LS (1994) Cytotoxic effects of gutta-percha solvents. J Endod 20, 6-8.

39. Beltes P, Koulaouzidou E, Kotoula V, Kortsaris AH (1995) In vitro evaluation of the cytotoxicity of calcium hydroxide-based root canal sealers. Endod Dent Traumatol 11, 245-249.

40. Torabinejad M, Parirokh M (2010) Mineral trioxide aggregate: A comprehensive literature review-Part II: Leakage and biocompatibility investigations. J Endod 36, 190-202.

41. Wang X, Jin T, Chang S, Zhang Z, Czajka-Jakubowska A, Nor JE et al. (2012) In vitro differentiation and mineralization of dental pulp stem cells on enamel-like fluorapatite surfaces. Tissue Eng Part C 18, 821-830.

42. Wang X, Zhang Z, Chang S, Czajka-Jakubowska A, Nor JE, Clarkson BH et al. (2014) Fluorapatite enhances mineralization of mesenchymal/endothelial cocultures. Tissue Eng Part A 20, 12-22.

43. Haglund R, He J, Jarvis J, Safavi KE, Spangberg LS, Zhu Q (2003) Effects of root-end filling materials on fibroblasts and macrophages in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 95, 739-745.

44. Anderson HC (1989) Mechanism of mineral formation in bone. Lab Invest 60, 320-330.

45. Liu J, Jin T, Chang S, Czajka-Jakubowska A, Zhang Z, Nor JE et al. (2010) The effect of novel fluorapatite surfaces on osteoblast-like cell adhesion, growth, and mineralization. Tissue Eng Part A 16, 2977-2986.

Table and figures

Table 1 Setting time (ST) and diametral tensile strength (DTS) of fluorapatite (FA)-forming calcium phosphate cement (CPC) with or without tricalcium silicate (TCS)

	ST (min)	DTS (MPa)
FA-forming CPC with TCS	10.3 ± 0.6^a	3.89 ± 0.76^a
FA-forming CPC without TCS	14.0 ± 1.0^b	2.65 ± 0.29^b

Mean$\pm$SD

In each analysis, values with a different lower-case letter are significantly different ($p<0.05$).
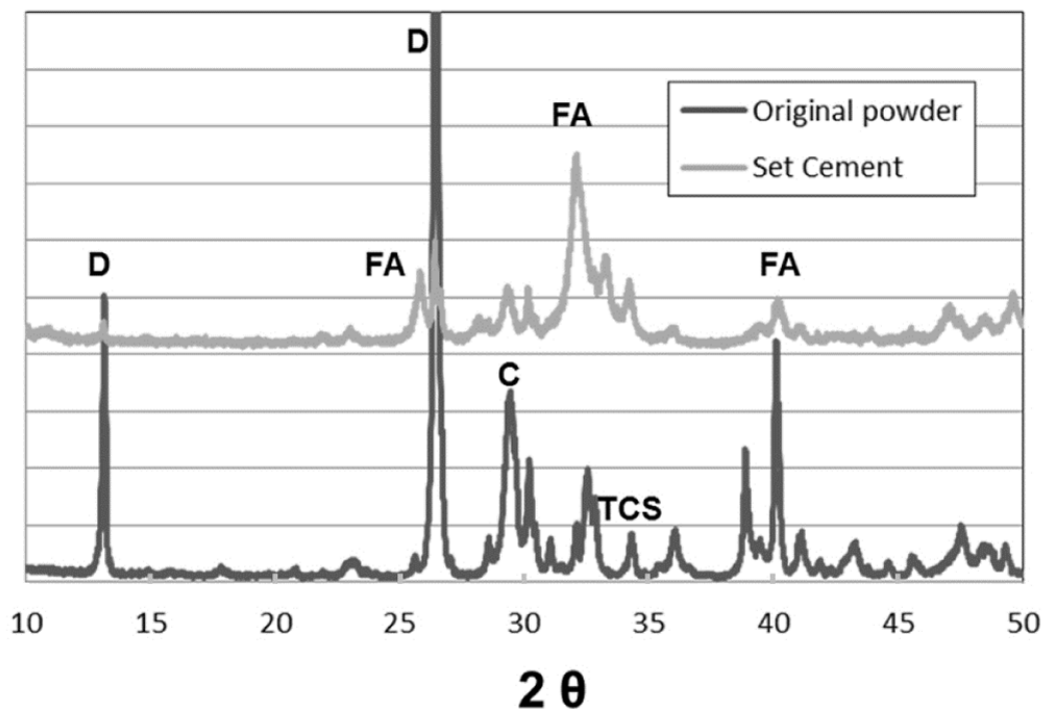
Fig. 1 XRD patterns of FA-forming CPC with TCS before and after setting.

FA: fluorapatite, D: DCPA, C: $CaCO_3$, TCS: tricalcium silicate
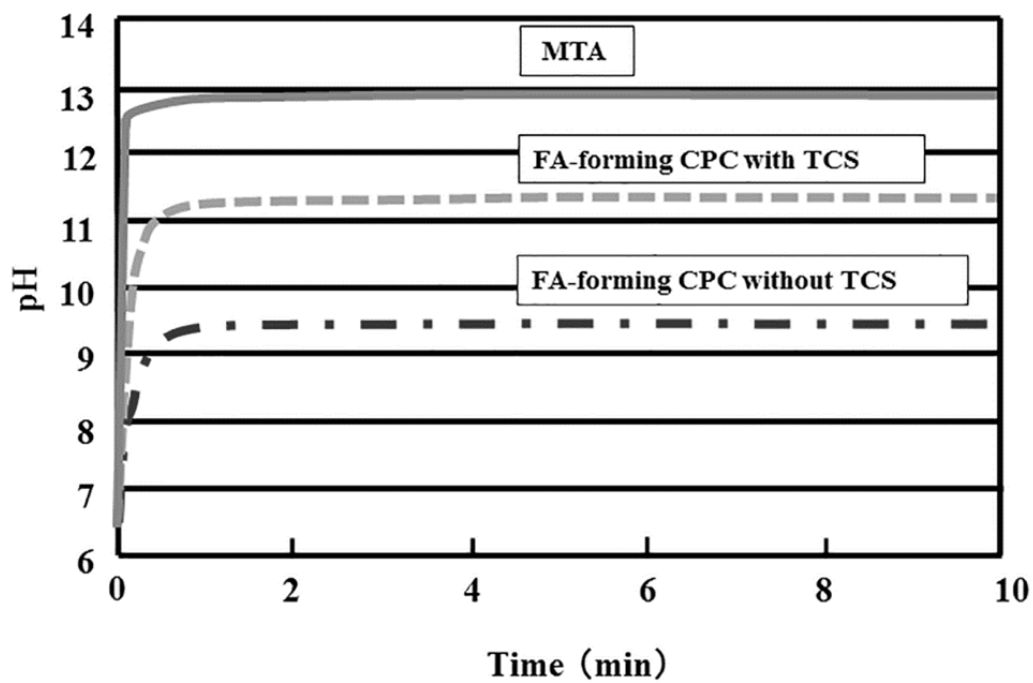
Fig. 2 The pH response curves of FA-forming CPC with and without TCS, and MTA.
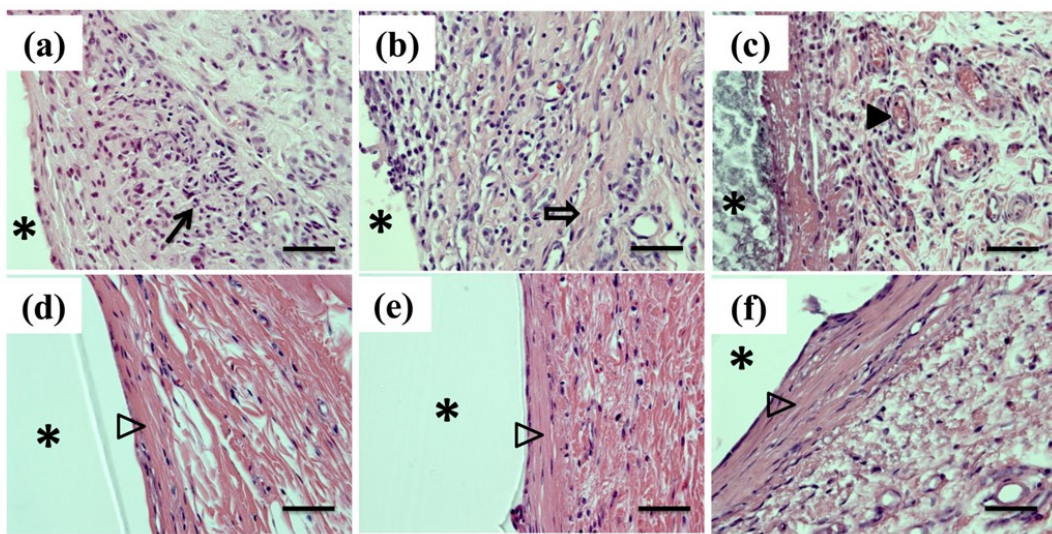
Fig. 3 Histolopathological findings of the tissue response at the open ends of polyethylene tubes (asterisks). At 7 (a–c) and 41 (d–f) days: the control group (a and d),FA-forming CPC with TCS group (b and e), and MTA group (c and f). Arrow: infiltration of inflammatory cells; open arrow: wavy collagen fiber deposits and fibrosis; arrowhead: vascular congestion; open arrowheads: thin fibrous capsule formations at the open ends of polyethylene tubes without infiltration of inflammatory cells (scale bars=50 mm).
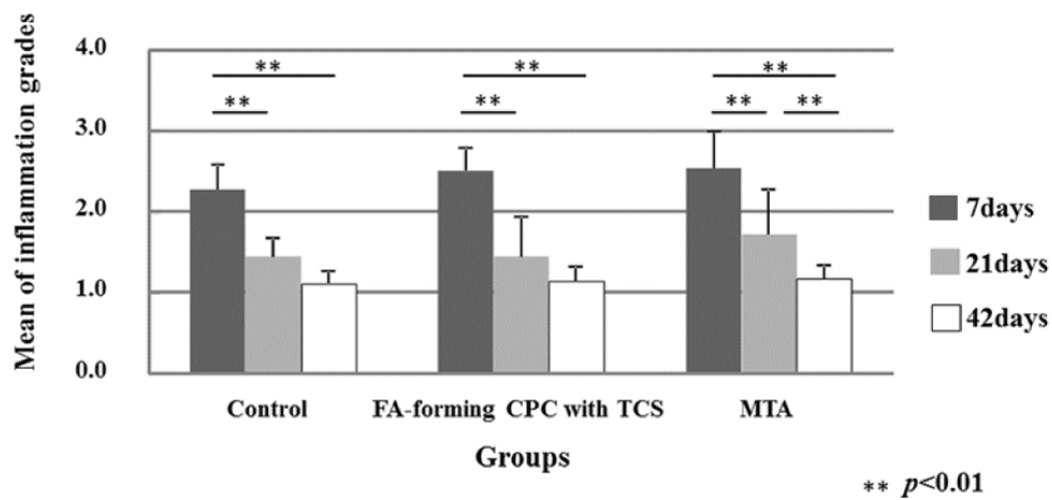
Fig. 4 Comparison of inflammation response in three groups at each test period
The inflammation grades in histopathological assessment were determined according to Cox and Robbin's criterion. Each value represents mean±SD (n=10). Values connected by a horizontal line are significantly different (**p<0.01).
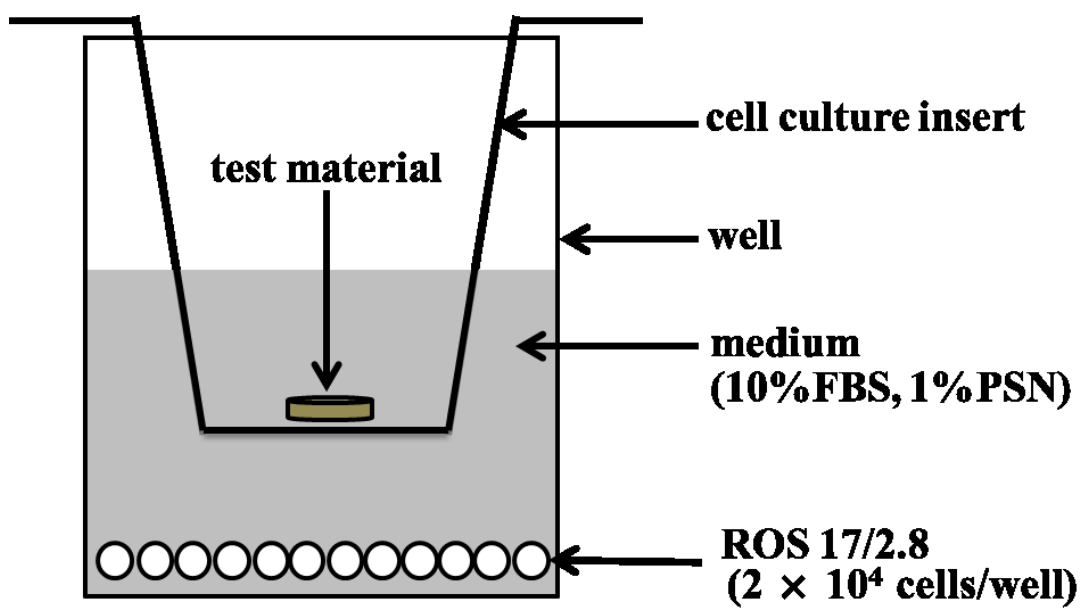
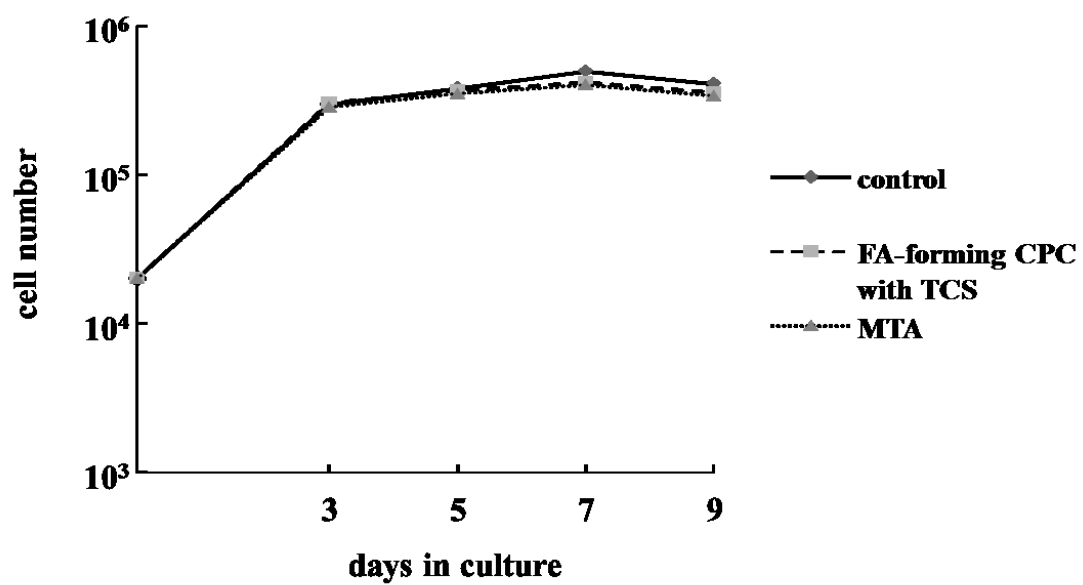Fig. 5 Schematic representation of a culture plate with a cell culture insert.

Fig. 6 Effect of FA-forming CPC with TCS, MTA, and control on proliferation of ROS 17/2.8 cells. Proliferation and viability of ROS cells after 3, 5, 7, and 9 days were determined using the Cell Counting Kit 8.
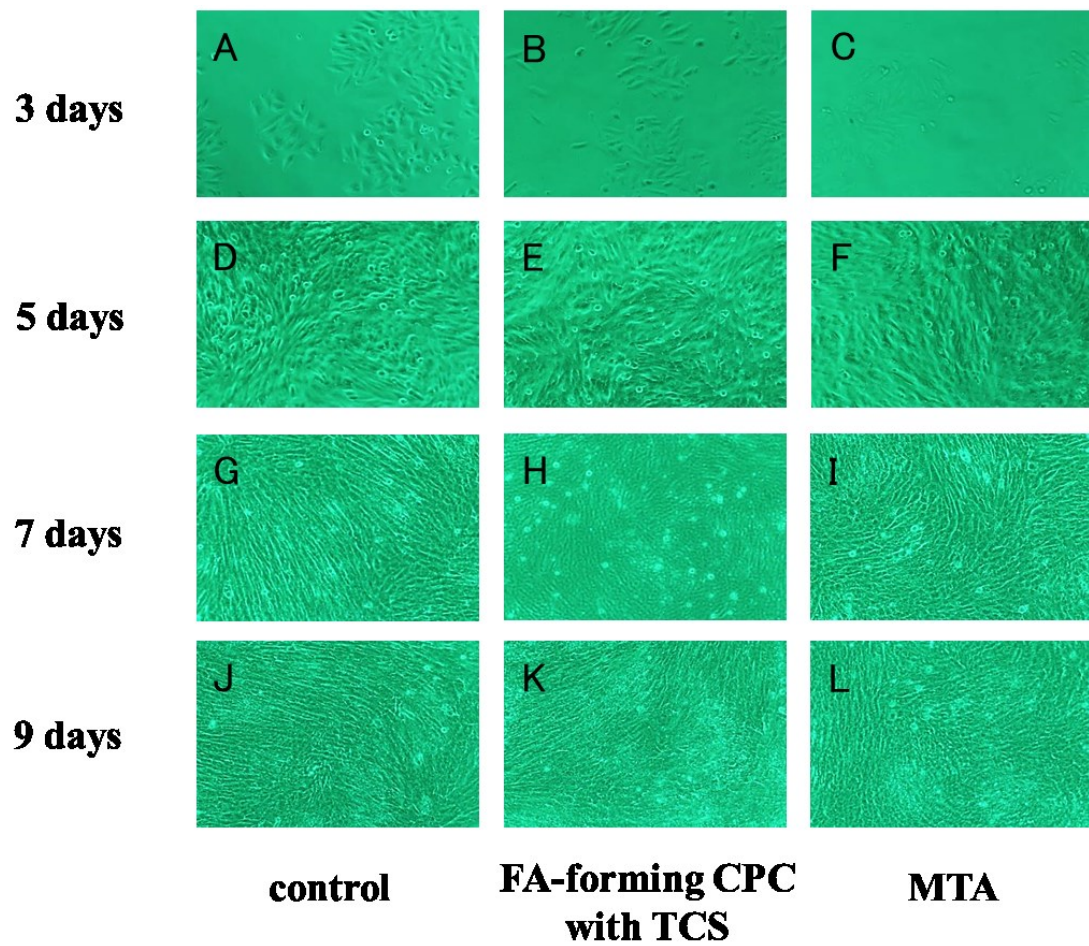
Fig. 7 Morphology of ROS 17/2.8 cells following incubation with FA-forming CPC with TCS, MTA, and control (original magnification, ×100).
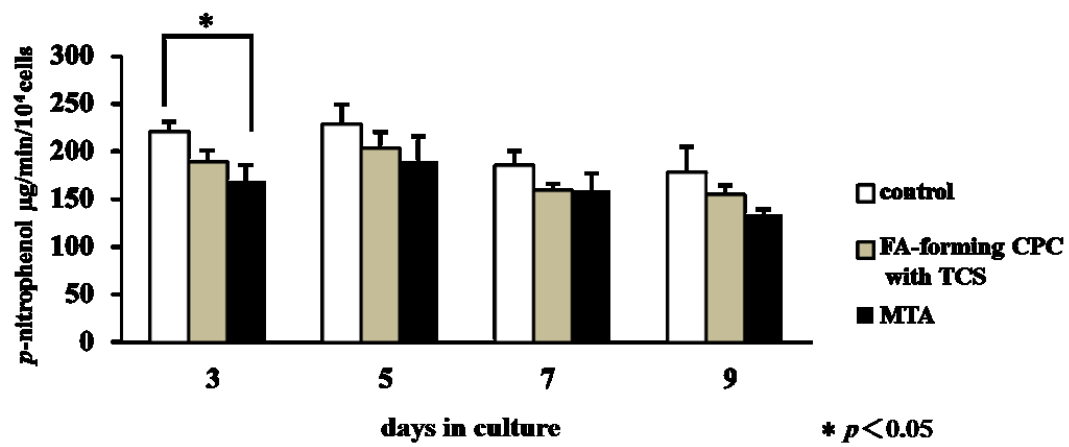
Fig. 8 Effects of FA-forming CPC with TCS, MTA, and the control on the ALP activity of ROS 17/2.8 cells. ALP activity was defined as the amount of p-nitrophenol produced per min. Each value represents the mean±standard deviation. *Significant difference ($p<0.05$).