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ORIGINAL ARTICLE

Effects of two fast-setting calcium-silicate cements on cell viability and angiogenic factor release in human pulp-derived cells

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Abstract Mineral trioxide aggregate (MTA) is considered a pulp-capping agent of choice, but has the drawback of a long setting time. This study aimed to assess two different types of calcium-silicate cements as pulp-capping agents, by investigating their in vitro cytotoxicity and angiogenic effects in human pulp cells. ProRoot MTA, Endocem Zr, and Retro MTA were prepared as set or freshly mixed pellets. Human pulp-derived cells were grown in direct contact with these three cements, Dycal, or no cement, for 7 days. Initial cell attachment, viability, calcium release, and the levels of vascular endothelial growth factor (VEGF), angiogenin, and basic fibroblast growth factor (FGF-2) were evaluated statistically using a linear mixed model ($P < 0.05$). The biocompatibility of Retro MTA was similar to those of the control and ProRoot MTA. Endocem Zr groups showed fewer and more rounded cells after a 3-day culture; however, the initial cytotoxicity appeared transient. All test materials showed significant increases in calcium concentration compared with the control group ($P < 0.05$). VEGF and angiogenin levels in ProRoot MTA and Retro MTA groups were significantly higher than those in the Endocem Zr group ($P < 0.05$). FGF-2 levels were not significantly different between groups ($P > 0.05$). We demonstrate that Retro MTA, which has a short setting time, has similar biocompatibility and angiogenic effects on human pulp cells, and can therefore potentially be as effective in pulp capping as ProRoot MTA. Endocem Zr showed intermittent cytotoxicity and elicited lower levels of VEGF and angiogenin expression.

Keywords Calcium-silicate cement · Fast setting · Mineral trioxide aggregate · Pozzolan cement · Pulp cell

Introduction

Mineral trioxide aggregate (MTA; ProRoot MTA; Dentsply Tulsa; Tulsa, OK, USA) has been widely used in endodontic treatments, because it has been proven to have biocompatible and odonto- and osteogenic qualities in a number of studies [1–5]. MTA, rather than calcium hydroxide, has become the material of choice for pulp capping, due to its superior outcomes [6, 7]. However, MTA has a long setting time, which is a drawback for clinicians during the pulp-capping procedure. In clinical situations, there is not enough space to apply MTA, wet cotton, and a proper temporary sealing between appointments.

Recently, fast-setting MTA-like calcium-silicate cements have been developed. Endocem Zr (Maruchi, Wonju, Korea) is an MTA-derived pozzolan cement and its initial setting time was reported to be 4 min in a recent study by Choi et al. [8]. Another fast-setting calcium silicate cement is Retro MTA® (bioMTA, Seoul, Korea), with an initial setting time of about 180 s, according to the manufacturer. These cements are relatively new and not many studies have investigated their

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Table 1 Major components of Endocem Zr and RetroMTA

Endocem Zr		RetroMTA	
	wt (%)		wt (%)
CaO	17–27	$CaCO_2$	60–80
SiO_2	7–11	SiO_2	5–15
Al_2O_3	3.5–4.7	Al_2O_3	5–10
ZrO_2	43–46	Ca–Zr complex	20–30
Fe_2O_3	1.3–2.3		
MgO	1.7–2.5		

biological effects on pulp cells. Their major components provided from the manufacturers are summarized in Table 1 and their short setting time may be due to the presence of zirconium, which has been reported to shorten the setting time by accelerating the hydration of Portland cement [9, 10].

Maintaining cell viability as well as enhancing angiogenesis after pulp capping is one of the critical aspects in the early stages of healing [11]. A pulp-capping agent should make direct contact with exposed pulp tissue. Therefore, investigation of the initial cell responses to a pulp-capping material may be important for proper wound healing and repair.

To enhance cell proliferation, it may be crucial to make use of material with reduced cytotoxicity. In addition, it is known that calcium ions present in the repaired tissue are involved in wound healing [12] and some capping materials are capable of promoting cell proliferation by increasing extracellular calcium levels [12, 13]. Extracts of tricalcium silicate (Ca_3SiO_5) have been found to enhance proliferation and differentiation of pulp cells [14, 15]. Since MTA and MTA-like cements contain calcium silicate, it may be possible to maintain a higher extracellular calcium concentration after these cements have been applied as a capping material [16]. Han et al. [10] demonstrated that an increased concentration of calcium ions is released from ProRoot MTA, Endocem, and Endocem Zr for up to 168 h.

Another critical step for successful outcomes is the formation of new vessels around the wound. In dental pulp, several angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietin, angiogenin, epidermal growth factor (EGF), and fibroblast growth factor (FGF) are present [11, 17]. The levels of these angiogenic growth factors are reported to be elevated in dental pulp in response to injury and inflammation [18, 19], and this phenomenon may be crucial for repair [11].

We aimed to evaluate the biological properties of these new MTA-like cements with a fast setting time in terms of cell viability and angiogenic potential, with this given background.

Materials and methods

Cell culture

The study protocol was approved by the institutional review board of the Gangnam Severance Hospital, Yonsei University, Seoul, Korea. Pulp cells were obtained from human premolars scheduled for extraction due to orthodontic treatment; these cells were cultured from the pulp specimens following the methods described in our previous study [20]. Cells obtained between passages 4 and 5 were used and they were characterized in our previous study [20]. Alpha modification of Eagle's medium (α-MEM; Invitrogen, Carlsbad, CA, USA), supplemented with 100 U/mL penicillin, 100 μL/mL streptomycin and 3 % fetal bovine serum (FBS) was used. The same number of cells (1×10^4 per cm^2) was seeded into 24-well plates that were coated with or without different types of calcium-enriched cements.

Material preparation

Powder (0.1 g) of tooth-colored ProRoot MTA, Retro MTA, and Endocem Zr was mixed based on the manufacturers' instructions. Then, the surface of a 24-well plate was coated with the mixture. To generate a consistent surface area covered with cement, a sterile Teflon tube (5-mm diameter and 3-mm thick) was used to apply the cement mixture to the cell culture plate. The cement-coated cell culture plate was incubated for 24 h at 37 °C under humidified conditions. We also used freshly mixed cements that were mixed and applied to the culture dishes immediately before adding cells, to mimic a clinical setting. Set ProRoot MTA, freshly mixed ProRoot MTA, set Retro MTA, fresh Retro MTA, set Endocem Zr, and fresh Endocem Zr are abbreviated as M(s), M(f), R(s), R(f), E(s), and E(f), respectively. Cell culture plates without a cement coating were used as control. When studying initial cell growth under the phase microscope, a cell culture grown on set Dycal® (Dentsply Caulk, Milford, DE, USA) was included as a positive control. Cement specimens for calcium measurements were prepared in the same manner and immersed into cell culture media in the absence of cells. Calcium concentration measured from cell culture media without any cement and cells was used as a baseline.

Phase and scanning electron microscopy

Cell attachment was examined under a phase microscope (Nikon TMS; Nikon Inc.; Melville, NY, USA) with 40× magnification after 3 and 7 days. For scanning electron microscopy (SEM), cells were fixed, washed, dehydrated using graded concentrations of ethanol, coated with 30-nm

gold particles, and observed under a scanning electron microscope (S-800; Hitachi; Tokyo, Japan) with 100–1000× magnification after 3 and 7 days of culture.

Cell viability test

Using 3- and 7-day cultures, cell viability was measured using an 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay (EZ-Cytox Cell Assay kit; Daeill Lab Service Co., Seoul, Korea), which measures cell dehydrogenase activity. This kit functions similarly to the 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay used for measuring mitochondrial activity in viable cells. The protocol used was as described in our previous study [21]. Two independent experiments were performed in quadruplicate.

Measurement of calcium ions released from cement into culture media

Calcium concentrations were measured with a Quantichrom™ calcium assay kit (Bioassay Systems, Hayward, CA, USA). Quantification of calcium was performed by assessing the optical density at 610 nm, using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA, USA) and Softmax Pro software (Molecular Devices). Each experiment was performed twice and each sample was analyzed in triplicate.

Measurement of angiogenic factors

The supernatant was collected from each cell culture plate at day 1 and 3 of culture, was centrifuged, and stored at −20 °C until required for further analysis. The amounts of VEGF, angiogenin, and FGF-2 were quantified in triplicate, using multiplex cytokine analysis with a Fluorokine MAP Multiplex assay kit (R&D Systems; Minneapolis, MN, USA) on a Luminex 200 instrument (Luminex; Austin, TX, USA). The data were analyzed with Bio-plex manager 6.1 software (Bio-Rad; Hercules, CA, USA).

Statistical analysis

Data were analyzed using linear mixed models (type III test of fixed effects) to detect statistically significant differences among groups and times. When the means of 2 groups were compared, P values were adjusted by Bonferroni correction. The Spearman correlation coefficient was calculated to evaluate the relationships between angiogenic factors. Statistical significance was set at $P < 0.05$.

Results

Cell attachment and viability

Cells in the control group that had been seeded without any cement attached to the culture plate well and showed a healthy pattern throughout experiments (Fig. 1a). In contrast, cells grown in the presence of Dycal pulp-capping agent were observed to be dead in 3- and 7-day cultures (Fig. 1b). After confirming that there were no viable cells in the Dycal group, we did not investigate Dycal in subsequent experiments. In the ProRoot MTA groups, both the M(s) and M(f) groups revealed well-attached cells, with no inhibition zone being observed around the cement at either day 3 or day 7 (Fig. 1c, d). In freshly mixed Retro MTA specimens (R(f)), no cell attachment could be observed by phase microscopy around the boundary of the cement (Fig. 1e) or by SEM at day 3 (Fig. 2c). This inhibition zone disappeared in the 7-day cultures, in which cells became flattened and attached well to the surface of the material (Fig. 2d). In R(s) samples, cells were healthy and attached well to the material surface. In the freshly mixed Endocem Zr group (E(f)), fewer cells could be observed by phase microscopy (Fig. 1g) and SEM revealed more rounded cells on the surface in the 3-day culture (Fig. 2e) In the 7-day culture of E(f), SEM revealed flattened cells (Fig. 2f).

There was a significant difference in cell viability among groups and times ($P = 0.0060$). In the 3-day cultures, there were fewer viable cells in the E(s) and E(f) groups than in the control and M(s) groups ($P < 0.05$). In the 7-day cultures, there was no statistically significant difference except for between M(f) and E(s) ($P = 0.031$). In each cement group, there was no significant difference between the set and fresh materials in terms of cell viability (Fig. 3).

Concentration of calcium ions released from test materials

After 24 h, the calcium concentrations in the cell culture media of the control, M(s), M(f), R(s), R(f), E(s), and E(f) groups were 2.48 (0.13), 2.9 (0.15), 3.55 (0.24), 3.02 (0.19), 3.24 (0.22), 2.50 (0.24), and 4.85 (0.15) mM, respectively. The statistically significant higher calcium concentrations were found in all freshly mixed cements compared with control. After 72 h, the calcium concentrations of the M(s), M(f), R(s), R(f), E(s), and E(f) groups were 3.13 (0.15), 4.64 (0.21), 4.24 (0.18), 4.54 (0.16), 4.50 (0.26), and 6.20 (0.27) mM, respectively, and the calcium concentrations of all experimental groups were higher than that of the control. Freshly mixed Endocem Zr (E(f)) released the highest concentrations of calcium ions at both times. The statistically significant differences between groups are marked in Fig. 4.

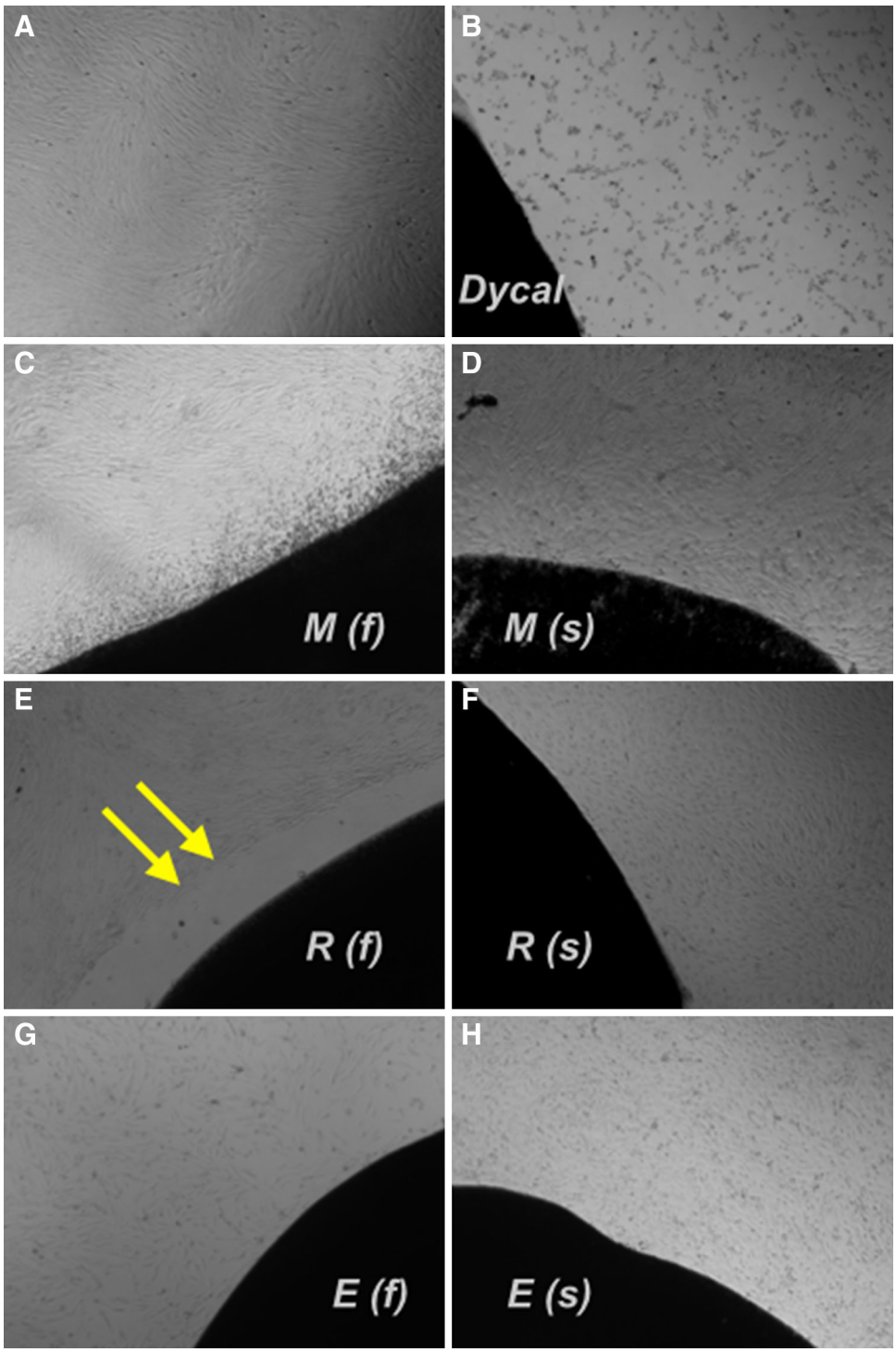

Fig. 1 Optical microscopy images (×40 magnification) of human pulp cells grown on a plastic surface (control) or pulp-capping cements for 3 days. **a** Cells grown on a plastic cell culture plate, **b** cells grown on Dycal were observed to be dead, **c** cells grown on fresh ProRoot MTA (M(f)), **d** cells grown on set ProRoot MTA (M(s)), **e** cells grown on fresh Retro MTA (R(f)). An inhibition zone (*arrows*) around the margin of the cement was observed, **f** cells grown on set Retro MTA(R(s)), **g** cells grown on fresh Endocem Zr (E(f)), **h** cells grown on set Endocem Zr (E(s))

ELISA measurement of released angiogenic growth factors

VEGF levels were measured in 24- and 72-h cultures for each of the test materials (Fig. 5a). There was a statistically significant difference in VEGF levels depending on the test materials and times used ($P = 0.0162$). There was no significant difference in VEGF levels between set and fresh ProRoot MTA groups ($P = 0.4594$), but there were differences between set and fresh Retro MTA and Endocem Zr ($P = 0.025$ and 0.0002, respectively). The Endocem Zr groups showed significantly lower concentrations of VEGF compared with the ProRoot MTA and Retro MTA groups.

Angiogenin levels of R(f), E(s), and E(f) at 24 h were significantly lower than that of the control (Fig. 5b). At 72 h, angiogenin levels in the M(s), M(f), E(s), and

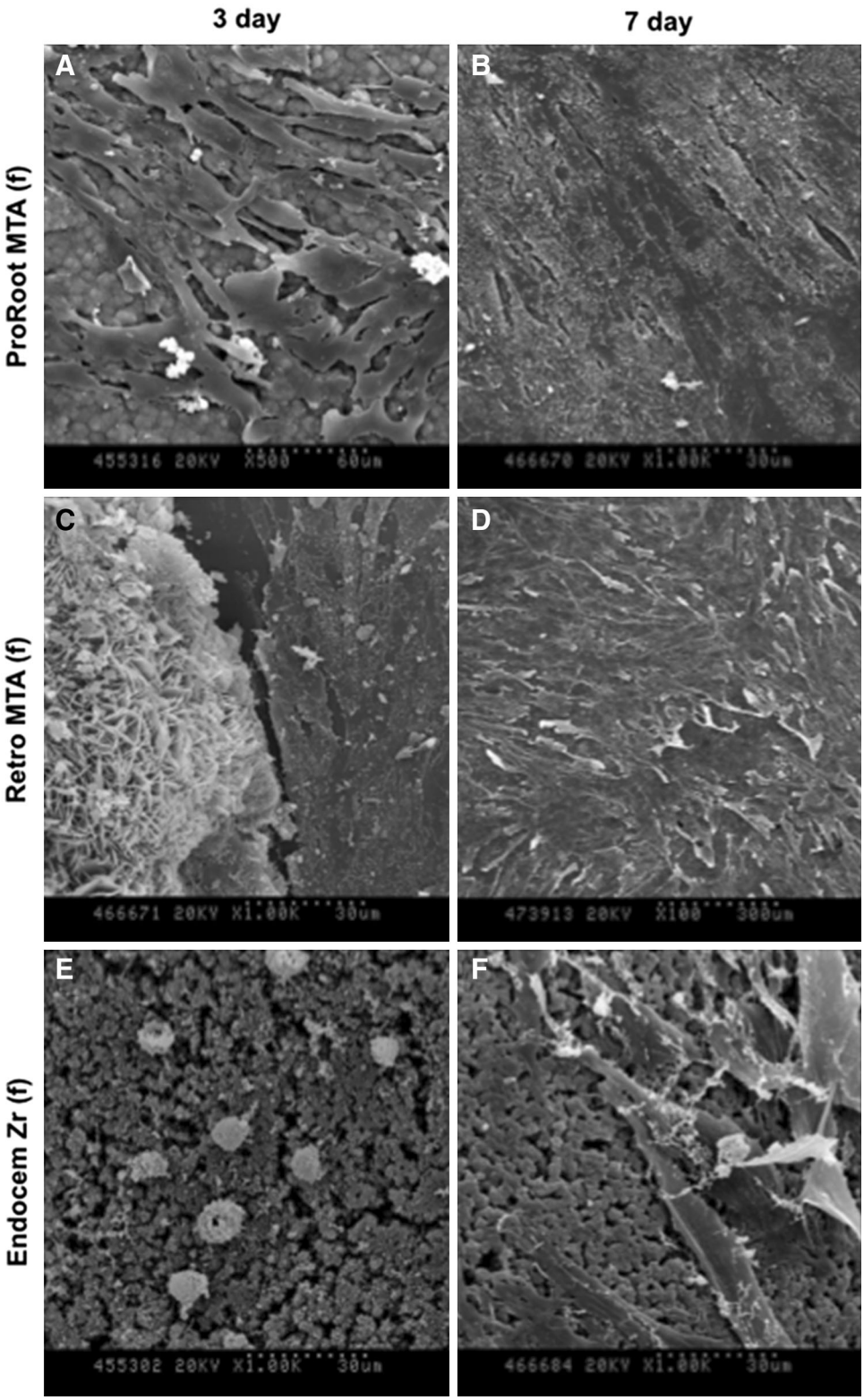

Fig. 2 Scanning electron microscopy (SEM) images of cell attachment after 3 and 7 days of growth on freshly prepared ProRoot MTA, Retro MTA, and Endocem Zr. **a** Cells grown on fresh ProRoot MTA for 3 days (×500), **b** cells grown on fresh ProRoot MTA for 7 days (×1000), **c** cells grown on fresh Retro MTA for 3 days (×1000), **d** cells grown on fresh Retro MTA for 7 days (×100), **e** cells grown on fresh Endocem Zr for 3 days (×1000), **f** cells grown on fresh Endocem Zr for 7 days (×1000)

E(f) groups were statistically significantly lower than that in the control (Fig. 5b). Levels of FGF-2 were not statistically significantly different between any groups (Fig. 5c).

A positive correlation was found between the levels of VEGF and angiogenin (Pearson coefficient = 0.69568, $P < 0.001$).

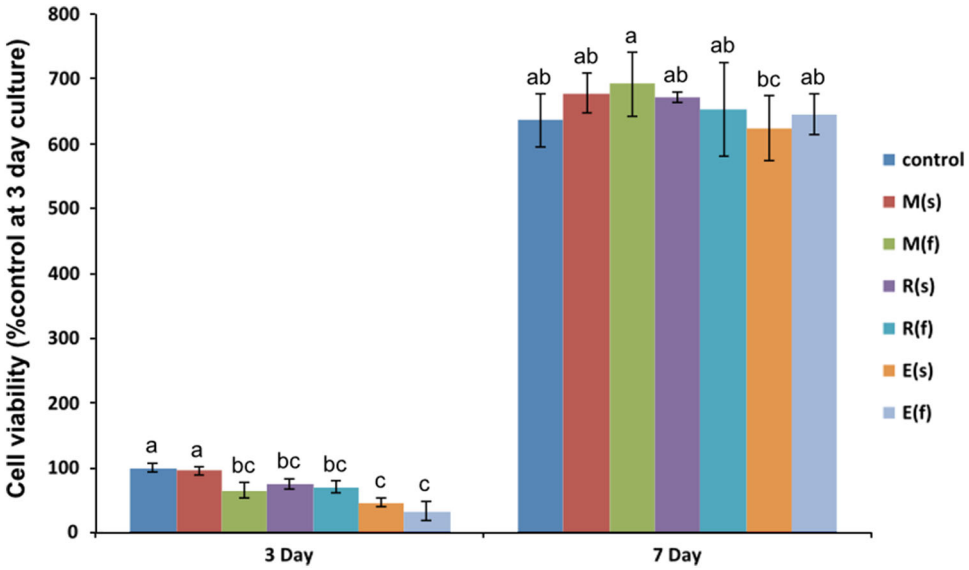

Fig. 3 Cell viability of 3- and 7-day cultures. M(s), set ProRoot MTA; M(f), freshly prepared ProRoot MTA; R(s), set Retro MTA; R(f), freshly prepared Retro MTA; E(s), set Endocem Zr; E(f), freshly prepared Endocem Zr. Data represent mean ± SD of quadruplicates. *Different letters* represent statistically significant differences between groups ($P < 0.05$)

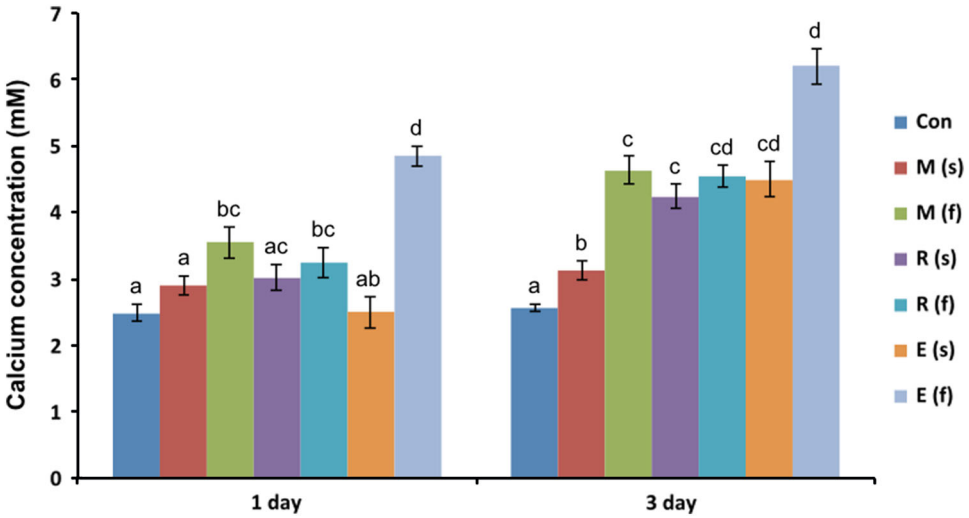

Fig. 4 Measurement of calcium concentrations at 24 and 72 h. Con, control (cell culture media), M(s), set ProRoot MTA; M(f), freshly prepared ProRoot MTA; R(s), set Retro MTA; R(f), freshly prepared Retro MTA; E(s), set Endocem Zr; E(f), freshly prepared Endocem Zr. Data represent mean ± SD of triplicates. *Different letters* represent statistically significant differences between groups ($P < 0.05$)

Discussion

We have investigated the initial responses of cells being placed in direct contact with pulp-capping agents in order to evaluate the biological properties of these new fast-setting cements. Phase microscopy, SEM, and XTT assays demonstrated that Endocem Zr might initially elicit cytotoxicity, particularly when it is freshly prepared; however, this phenomenon appeared to be transient. These findings are consistent with those of the study by Song et al. [22], which demonstrated the initial cytotoxicity of freshly prepared Endocem cement in 48-h cultures. Other previous studies on Endocem used extracts of the cement [8, 16] and reported no significant differences in cell viability among control, ProRoot MTA, and Endocem cultures. Endocem Zr is a tooth-colored version of Endocem and both cements are derived from Pozzolan cement. A study by Han et al. [23] showed a higher concentration of aluminum in Endocem Zr than in ProRoot MTA; moreover, aluminum can cause cytotoxicity. Aluminum in Endocem Zr may affect initial cell growth, however, this assumption is not confirmed since Retro MTA also contains Al_2O_3. Retro MTA showed similar effects to ProRoot MTA on cell attachment and viability. To our knowledge, no previous publication has investigated the properties of Retro MTA. Instead, Ortho MTA (bioMTA), which is known to have similar components, has been studied recently [24, 25]. Lee et al. [24] reported that Ortho MTA was less biocompatible than ProRoot MTA or glass ionomer cement; this was inconsistent with the findings of our study. Another study also indicated that Ortho MTA demonstrated similar biocompatibility and effects on cell differentiation of dental pulp cells as Biodentine and MTA-angelus [26].

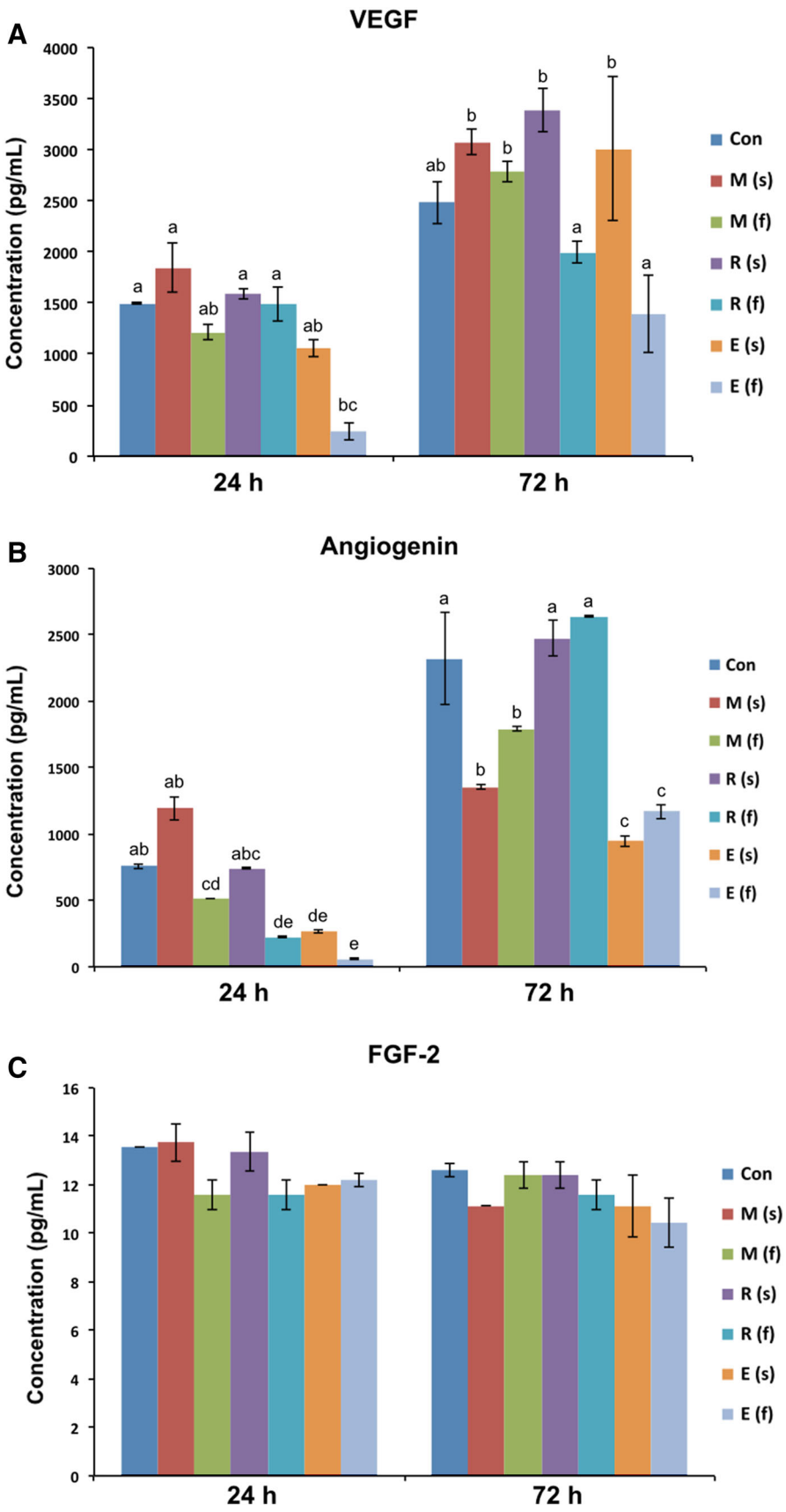

Fig. 5 Levels of VEGF, angiogenin, and FGF-2 released from cells grown in the presence of various pulp-capping materials at 1 and 3 days. **a** VEGF levels, **b** Angiogenin levels, **c** FGF-2 levels. M(s), set ProRoot MTA; M(f), freshly prepared ProRoot MTA; R(s), set Retro MTA; R(f), freshly prepared Retro MTA; E(s), set Endocem Zr; E(f), freshly prepared Endocem Zr. Data represent mean ± SD of triplicates. *Different letters* represent statistically significant differences between groups ($P < 0.05$)

Next, we measured the calcium ions released from the cement pellets after 72 h, since the extracellular calcium concentration can affect pulp cell proliferation and differentiation [15, 27]. Fresh cement, regardless of the type, released more calcium ions than set cements, and, among all groups, the fresh Endocem Zr group showed the highest calcium concentration in cell culture media at both day 1 and 3 at each time point. In a previous study by Park et al. [16], the percentages of calcium released by ProRoot MTA and Endocem were both around 40 %. The increased calcium ions released from cement pellets may affect cell proliferation. Under cell culture conditions, the optimum calcium concentration in the culture media of osteogenic cells is known to be around 1.8–2.2 mM [13]. In our study, the calcium concentration was increased up to 6.2 mM in freshly mixed Endocem Zr samples and this extremely high calcium concentration in the cell culture media can cause further cell death [28]. Since our study did not measure the extracellular calcium concentration during culture of pulp cells, the possible implications of high calcium on cell viability should be investigated in further studies.

To investigate the angiogenic factors released from cells exposed to cement, three angiogenic growth factors were evaluated in the study. We measured the levels of these angiogenic factor levels at 24 and 72 h, because the dental pulp cells tend to release the angiogenic factors early after trauma [19]. VEGF is known to be a potent angiogenic growth factor [29] and has been detected in human dental pulp [30]. It has also been shown to enhance proliferation and differentiation in dental pulp cells [31]. In this study, the M(s) group showed a higher concentration of VEGF than did the control group at both time points; however, there were no statistically significant differences between these two groups. This finding is partly in agreement with the study by Paranjpe et al. [32], which reported a 1.7-fold increase in the secretion of VEGF from pulp cells grown in the presence of MTA. At both times, VEGF levels of the ProRoot MTA and Retro MTA groups were higher than those of the Endocem Zr group. These findings could be interpreted in 2 ways. First, Endocem Zr triggered decreased amounts of angiogenic factors from pulp cells, or second, due to fewer viable cells in the Endocem Zr, this pulp-capping agent could cause reduced levels of VEGF. Interestingly, the viability of cells in the E(f) group was 33.8 % of that of the control at 72 h, while the level of VEGF in this group was 56.0 % of that in the control group.

We also measured angiogenin and FGF-2 levels. Angiogenin is involved in stimulating endothelial cells, particularly during neovascularization [29], while FGF-2 has been reported to promote cell differentiation and calcification in human pulp cells [33]. Although angiogenin levels did not increase after exposure to any type of cement in this study, pulp cells grown in the presence of cement released a particular level of angiogenin, regardless of the time point. However, the concentration of FGF-2 was relatively low in all groups and there was no statistically significant difference between groups.

In the present study, we did not investigate odontogenic differentiation of pulp cells in the presence of these cements, which could also be a crucial aspect for evaluating a material as a pulp-capping agent. Recently, Chang et al. [25] showed that ortho MTA, which is a similar type of retro MTA, had an enhancing effect on odontogenic differentiation. In previous studies [8, 16], it has been demonstrated that odontogenic-related markers were highly expressed in both MTA- and Endocem-treated groups. Given these findings, it can be inferred that the initial toxicity of Endocem Zr demonstrated in our study does not adversely affect the capability of pulp cells to differentiate.

Our study indicated that ProRoot MTA and Retro MTA had similar biological effects on human pulp cells, while Endocem Zr demonstrated transient cytotoxicity; these findings demonstrate the possibility that Retro MTA can be applied as an effective pulp-capping agent.

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Conflict of interest The authors deny any conflicts of interest.

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