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Biocompatibility and Hard Tissue-forming Ability of CPP-ACP- and CPP-ACFP-modified Calcium Silicate-based Cements

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Abstract

Aims: To evaluate the biocompatibility and osteogenic potential of calcium silicate-based cements (CSCs) modified with casein phosphopeptide – amorphous calcium phosphate (CPP-ACP) and casein phosphopeptide – amorphous calcium fluoride phosphate (CPP-ACFP). **Materials and method**: Commercially available CSCs were modified with CPP-ACP or CPP-ACFP to prepare CPP-ACP and CPP-ACFP-modified Biodentine™ (0%, 0.5%, 4.0% w/w), Angelus® MTA (0%, 0.5%, 2.0% w/w) and NEX® MTA (0%, 0.5%, 3.0% w/w). For each group, 50 mg, 300 mg and 1500 mg of the cement mixed according to manufacturers' instructions were placed and adapted at the bottom of 96 well, 24 well and 6 well (respectively) cell culture plates. After 24 h, the cement-coated plates were sterilised by ultraviolet light for 1 h. MG-63, MC3T3-E1, HGF-1, NIH3T3 cells were grown in the cement-coated plates and the cellular proliferation, cellular toxicity, alkaline phosphatase activity, cytokine production (interleukin-1α; IL-1α and interleukin-6; IL-6) and expression of mineralisation-associated proteins (collagen type 1, osteocalcin and osteopontin) were determined. **Results**: The addition of 0.5% CPP-ACP and 4.0% CPP-ACFP to Biodentine™, and 2.0% CPP-ACFP to Angelus® MTA significantly reduced the proliferation of MG-63. The addition of 4.0% CPP-ACP and 4.0% CPP-ACFP to Biodentine™, 2.0% CPP-ACFP to Angelus® MTA and 3.0% CPP-ACFP to NEX® MTA significantly reduced the proliferation of MC3T3-E1 cells. The tested cements, with and without CPP-ACP and CPP-ACFP, did not induce cellular toxicity nor IL-1α release. The addition of CPP-ACP and CPP-ACFP to Biodentine™ and NEX® MTA, and the addition of CPP-ACP to Angelus® MTA significantly increased the alkaline phosphatase activity of MG-63 cells. The presence of 4.0% CPP-ACP in Biodentine™, 0.5% and 2.0% CPP-ACP in Angelus® MTA, 0.5% CPP-ACFP in Angelus® MTA, and 0.5% CPP-ACFP in NEX® MTA significantly increased the alkaline phosphatase activity of MC3T3-E1 cells. All the tested cements significantly increased the release of IL-6 from MG-63 compared with negative control. The presence of CPP-ACP and CPP-ACFP in NEX® MTA significantly increased the release of IL-6 from MG-63 compared with unmodified NEX® MTA. MC3T3-E1 cells grown on Biodentine™ (unmodified and modified groups) and 3.0% CPP-ACFP-modified NEX® MTA released significantly higher IL-6 compared with negative control. 0.5% CPP-ACFP-modified Biodentine™ and 3.0% CPP-ACFP-modified NEX® MTA induced significantly higher IL-6 release from MC3T3-E1 compared with unmodified Biodentine™ and unmodified NEX® MTA respectively. The

tested cements (especially Biodentine™, Angelus® MTA and CPP-ACFP-modified cements) induced the secretion of mineralisation-associated proteins (especially collagen type 1 and osteocalcin).**Conclusions**: The tested cements are biocompatible, and they could serve as a suitable scaffold which supports cellular proliferation. The addition of CPP-ACP and CPP-ACFP to CSCs improved the cements' potential to induce osteoblastic differentiation.

التوافق الحيوي والقدرة على تكوين الأنسجة الصلبة لـ CPP-ACP- وCPP-ACFP- الأسمنت المعتمد على سيليكات الكالسيوم المعدل بـ

الملخص

الأهداف: تهدف الدراسة الى تقييم التوافق الحيوي وإمكانات تكوين العظم للأسمنت القائم على سيليكات الكالسيوم (CSCs) المعدل باستخدام فوسفوبيتيد الكازين - فوسفات الكالسيوم غير المتبلور (CPP-ACP) وفوسفوبيبتيد الكازين - فوسفات فلوريد الكالسيوم غير المتبلور (CPP-ACFP). **المواد وطرائق العمل**: تم تعديل الخلايا الجذعية السرطانية المتاحة تجارياً باستخدام CPP-ACP أو CPP-ACFP لإعداد Biodentine™ المعدل CPP-ACP وCPP-ACFP (0%، 0.5%، 4.0% وزن/وزن)، Angelus® MTA (0% ، 0.5%، 2.0% وزن/وزن) وNEX® MTA (0%، 0.5%، 3.0% وزن/وزن). لكل مجموعة، تم وضع 50 ملجم، 300 ملجم و1500 ملجم من الأسمنت المخلوط وفقًا لتعليمات الشركات المصنعة وتكييفها في قاع 96 بئرًا و24 بئرًا و6 لوحات زراعة خلايا بئر (على التوالي). بعد 24 ساعة، تم تعقيم الألواح المطلية بالأسمنت بواسطة الأشعة فوق البنفسجية لمدة ساعة واحدة. تمت زراعة خلايا MG-63، MC3T3-E1، HGF-1، NIH3T3 في الصفائح المطلية بالأسمنت والتكاثر الخلوي، والسمية الخلوية، ونشاط الفوسفاتيز القلوي، وإنتاج السيتوكينات (interleukin-1α؛ IL-1α وinterleukin-6؛ IL-6) وتم تحديد التعبير عن البروتينات المرتبطة بالتمعدن (نوع الكولاجين 1، أوستيوكالسين وأوستيوبونتين).**النتائج**: أدت إضافة 0.5% CPP-ACP و4.0% CPP-ACFP إلى Biodentine™، و2.0% CPP-ACFP إلى Angelus® MTA إلى تقليل انتشار MG-63 بشكل كبير. أدت إضافة 4.0% CPP-ACP و4.0% CPP-ACFP إلى Biodentine™، و2.0% CPP-ACFP إلى Angelus® MTA و3.0% CPP-ACFP إلى NEX® MTA إلى تقليل تكاثر خلايا MC3T3-E1 بشكل كبير. الأسمنت الذي تم اختباره، مع أو بدون CPP-ACP وCPP-ACFP، لم يسبب سمية خلوية ولا إطلاق IL-1α. أدت إضافة CPP-ACP وCPP-ACFP إلى Biodentine™ وNEX® MTA، وإضافة CPP-ACP إلى Angelus® MTA إلى زيادة نشاط الفوسفاتيز القلوي لخلايا MG-63 بشكل ملحوظ. أدى وجود CPP-ACP 4.0% في Biodentine™، و0.5% و2.0% CPP-ACP في Angelus® MTA، و0.5% CPP-ACFP في Angelus® MTA، و0.5% CPP-ACFP إلى زيادة كبيرة في الفوسفاتيز القلوي. نشاط خلايا MC3T3-E1. أدت جميع الأسمنتات التي تم اختبارها إلى زيادة تحرر IL-6 من MG-63 بشكل ملحوظ مقارنة بالتحكم السلبي. أدى وجود CPP-ACP وCPP-ACFP في NEX® MTA إلى زيادة كبيرة في إطلاق IL-6 من MG-63 مقارنةً بـــ NEX® MTA غير المعدل. أطلقت خلايا MC3T3-E1 المزروعة على Biodentine™ (مجموعات غير معدلة ومعدلة) وNEX® MTA المعدلة بـ 3.0% من CPP-ACFP معدل IL-6 أعلى بكثير مقارنة بالتحكم السلبي. أدى Biodentine™ المعدل بـــ CPP-ACFP بنسبة 0.5% وNEX® MTA المعدل بـــ CPP-ACFP بنسبة 3.0% إلى إطلاق IL-6 أعلى بكثير من MC3T3-E1 مقارنةً بـــ Biodentine™ غير المعدل وNEX® MTA غير المعدل على التوالي. تسببت الأسمنتات التي تم اختبارها (خاصة Biodentine™ وAngelus® MTA والأسمنت المعدل بـــCPP-ACFP) في تحفيز إفراز البروتينات المرتبطة بالتمعدن (وخاصة الكولاجين من النوع 1 والأوستيوكالسين)..**الاستنتاجات**: تعتبر انواع السمنت التي تم اختبارها متوافقة حيويا، ويمكن أن تكون بمثابة سقالة مناسبة تدعم التكاثر الخلوي. أدت إضافة CPP-ACP وCPP-ACFP إلى الخلايا الجذعية السرطانية إلى تحسين قدرة الأسمنت على تحفيز التمايز العظمي

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INTRODUCTION

Calcium silicate-based cements (CSCs), such as mineral trioxide aggregate (MTA), are endodontic repair materials with a wide range of clinical applications, including pulp capping, pulpotomy, perforation repair, apexification procedures and root-end filling.[1] CSCs are multipurpose biomaterials with an ability to release calcium hydroxide which dissociates in an aqueous environment into calcium ions (Ca2+) and hydroxyl ions (OH–), contributing to its mechanism of action in hard-tissue deposition and antibacterial activity [2]. Ca2+ ions released by CSCs enhance hard tissue-forming cell (odontoblast, cementoblast, osteoblast) viability, proliferation and differentiation and the OH– increase the alkalinity of the environment which is unfavourable for bacterial growth.[3, 4] The OH– ions produce an alkaline environment that supports pulpal repair, active calcification and neutralization of lactic acid produced by osteoclasts, consequently preventing dissolution of the mineral constituents of dentine; the alkaline pH could also activate alkaline phosphatase (ALP) enzyme that has a significant role in hard tissue formation.[5] The capability of CSCs to stimulate differentiation of the hard tissue cells, which is conducive to hard tissue formation, has been demonstrated in vitro. The expression of osteogenic phenotypes such as ALP, osteocalcin, bone sialoprotein, and osteopontin was shown after incubation of osteoblast-like cell line with CSCs.[6] The hard tissue forming activity of MTA is also attributed to its ability to release an abundance of Ca2+, which interacts with phosphate ions (PO43-; Pi) in the surrounding tissue fluid to form apatite-like precipitates on the surface of MTA. [7] The ability of an endodontic cement to form apatite-like crystals may induce odontoblastic differentiation and reparative dentine formation.[8]

Casein phosphopeptide – amorphous calcium phosphate (CPP-ACP) is manufactured by tryptic digestion of the milk protein casein then aggregation with Ca2+ and Pi and purification by ultrafiltration.[9] CPP-ACP has been used in dental practice for the inhibition of demineralisation and promotion of remineralisation of enamel and dentine, acid buffering capacity and biofilm-moderating effect.[10] A fluoride-containing version of CPP-ACP, known as CPP-ACFP (casein phosphopeptide –amorphous calcium fluoride phosphate) was also introduced as a remineralising agent with superior effect in comparison with CPP-ACP. [11] The biocompatibility of CPP-ACP along with its bioactive behaviour by providing Ca2+ and Pi for the mineralisation process in addition to the ability of CPP, enriched with Ca2+, to promote osteoblastic differentiation justifies considering CPP-ACP as a bioactive additive to improve the performance of the endodontic repair materials used in vital pulp therapy and

apexification procedures.[12, 13] The ability of CPP-ACP-modified CSCs to release Ca2+, Pi and OH– may provide the basic requirements for hydroxyapatite formation.[14] The present study aimed to investigate the biocompatibility and ion release of CPP-ACP- and CPP-ACFP-modified CSCs. The aim of this study was to investigate the effects of CPP-ACP-modified and CPP-ACFP-modified CSCs on the cellular proliferation, cytotoxicity and hard tissue formation.

MATERIALS AND METHODS

Ca^{2+} release and determination of CPP-ACP and CPP-ACFP amount added to each cements

Commercially available Biodentine™ (BD; Septodont, Saint Maur des Fosses, France), Angelus® MTA (AMTA; Angelus Industria de Produtos Odontologicos Ltda., Londrina, PR, Brazil), and NEX® MTA (NMTA; GC Corp, Tokyo, Japan) were tested in the present study. To investigate Ca^{2+} ion release, various amounts of CPP-ACP were mixed with the powder of the test materials. For each cement, six groups with different CPP-ACP concentrations were tested (0%, 1.0%, 2.0%, 4.0%, 8.0%, 16.0% w/w CPP-ACP). An analytical balance, accurate to 0.1 mg (Precisa Gravimetrics AG, Dietikon, Switzerland), was used to weigh the powder of the cements and CPP-ACP. The cements were prepared according to the manufacturers' instructions. For each group, 50 mg of mixed cement was placed and adapted at the bottom of a 96 well cell culture plate. The cements were allowed to set for 24 h at 37°C and relative humidity of at least 95%. Freshly prepared 0.9% w/v sodium chloride solution (300 µl) was added to the wells containing the set cements. After 3 d of storage at 37°C and relative humidity of at least 95%, Ca^{2+} released into the storage solutions was measured by atomic absorption spectroscopy (Varian Australia Pty. Ltd., Mulgrave, Victoria, Australia). Three replicate samples were tested for each group (n = 3).

According to the findings of our previous study [15], 0.2% - 1.5% (w/v) CPP-ACP and 0.2% - 1.5% w/v CPP-ACFP solutions induced osteoblastic differentiation determined by improving the ALP activity. In a neutral pH environment, 0.2% w/v CPP-ACP and CPP-ACFP solutions released approximately 0.3 mM of Ca^{2+} (11), and therefore 1.5% w/v CPP-ACP and CPP-ACFP solutions may release about 1.9 mM of Ca^{2+}. The results of Ca^{2+} release (Table 1) showed that 1.0% w/w CPP-ACP-modified cements increased the release of Ca^{2+} by almost 0.6 mM compared with unmodified cements. 4.0% w/w CPP-ACP-modified BD increased the release of Ca^{2+} by almost 1.8 mM compared with unmodified BD. 2.0% w/w CPP-ACP-modified AMTA increased the release of Ca^{2+} by almost 1.8 mM compared with unmodified AMTA. It was also estimated that 3.0% w/w CPP-ACP-modified NMTA may increase the release of Ca^{2+} by almost

1.8 mM compared with unmodified NMTA. Depending on these results and to achieve approximately 0.3 mM increase in Ca^{2+} release, the cements were modified by 0.5% w/w CPP-ACP or 0.5% w/w CPP-ACFP. To increase Ca^{2+} release by approximately 1.8 mM, BD was modified by 4.0% w/w CPP-ACP or CPP-ACFP, AMTA was modified by 2.0% w/w CPP-ACP or CPP-ACFP and NMTA was modified by 3.0% w/w CPP-ACP or CPP-ACFP to perform the subsequent cell culture assays.

Table 1: Calcium ion release (mM) from CPP-ACP-modified CSCs (placed at the bottom of 96 well-plates) into 0.9% sodium chloride solution after 3 days storage.

	Biodentine™	Angelus® MTA	NEX® MTA
0% CPP-ACP	6.3	2.4	3.9
1% CPP-ACP	6.9	3	4.5
2% CPP-ACP	7.5	4.2	5.4
4% CPP-ACP	8.1	6	6.3
8% CPP-ACP	8.7	7.2	8.7
16% CPP-ACP	10.5	10.5	10.8

CPP-ACP and CPP-ACFP-modified CSCs for cell culture assays

According to the previous Ca^{2+} release results and estimations done above, the powders of the test cements were modified with CPP-ACP and CPP-ACFP as follows: for BD, unmodified BD, 0.5% w/w and 4.0% w/w CPP-ACP-modified BD, and 0.5% w/w and 4.0% w/w CPP-ACFP-modified BD were tested. For AMTA, unmodified AMTA, 0.5% w/w and 2.0% w/w CPP-ACP-modified AMTA, and 0.5% w/w and 2.0% w/w CPP-ACFP-modified AMTA were tested. For NMTA, unmodified NMTA, 0.5% w/w and 3.0% w/w CPP-ACP-modified NMTA, and 0.5% w/w and 3.0% w/w CPP-ACFP-modified NMTA were tested. The unmodified and modified cements were prepared according to the manufacturers' instructions. For each group, 50 mg, 300 mg and 1500 mg of mixed cement were placed and adapted at the bottom of 96 well, 24 well and 6 well (respectively) cell culture plates. The cements were allowed to set for 24 h at 37°C and relative humidity of at least 95%. After setting, the cement-coated plates were sterilised by ultraviolet light for 1 h before starting the assays.

Cell culture

Human (MG-63) and mouse (MC3T3-E1) osteoblast-like cell lines (Sigma-Aldrich, Castle Hill NSW, Australia), and human (HGF-1) and mouse (NIH3T3) fibroblast-like cell lines (ATCC, Manassas, VA, USA) were cultured in complete Alpha Minimum Essential Medium (α-MEM), composed of α-MEM supplemented with 10% (v/v) foetal calf serum (FCS), 1 U/ml penicillin and 0.1 mg/ml streptomycin, 1 mM sodium pyruvate and 2 mM L-Glutamine (Sigma-Aldrich, Castle Hill NSW, Australia). For the mineralisation assay, the complete α-MEM was supplemented with 10 mM glycerophosphate and 50 µg/ml ascorbic acid (Sigma-Aldrich, Castle Hill NSW, Australia) to produce osteogenic media. Cells were maintained in a humidified Heracell 150 incubator (ThermoFisher Scientific™, Rockford, IL, USA) at 37ºC and 5% (v/v) CO_2.

Cell proliferation and cell cytotoxicity assays

MG-63, MC3T3-E1, HGF-1 and NIH3T3 cells were sub-cultured in 96 well culture plates (containing the unmodified and CPP-ACP and CPP-ACFP-modified BD [0%, 0.5%, 4.0% w/w], AMTA [0%, 0.5%, 2.0% w/w] and NMTA [0%, 0.5%, 3.0% w/w]) with complete α-MEM (5×10^3 cell/well in 300 µl) and incubated for 3 d. Cells cultured with complete α-MEM alone (without cement) were used as the negative control group. Three replicate samples were tested for each group (n = 3). Cell proliferation and cell cytotoxicity were assessed using methyl-thiazol-tetrazolium assay kit (MTT, CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay) and lactate dehydrogenase assay kit (LDH, CytoTox 96® Non-Radioactive Cytotoxicity Assay) respectively, according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

Alkaline phosphatase activity and cytokine production

MG-63, MC3T3-E1, HGF-1 and NIH3T3 cells were sub-cultured in 24 well culture plates (containing the unmodified and CPP-ACP and CPP-ACFP-modified BD [0%, 0.5%, 4.0% w/w], AMTA [0%, 0.5%, 2.0% w/w] and NMTA [0%, 0.5%, 3.0% w/w]) with complete α-MEM (5×10^4 cell/well in 2 ml). Cells cultured with complete α-MEM alone (without cement) were used as the negative control group. Cells cultured with complete α-MEM modified with 20 ng/ml *Escherichia Coli* lipopolysaccharide (LPS, Invitrogen, San Diego, CA, USA) were used as the positive control for cytokines release. Three replicate samples were prepared for each group (n = 3).

After 3 d, the supernatant was collected and stored in a –80°C freezer (ThermoFisher Scientific™, Rockford, IL, USA) until use for measurement of cytokine production. The cultured osteoblast-like cells (MG-63 and MC3T3-E1) were washed with phosphate buffer saline (PBS, Sigma-Aldrich, Castle Hill NSW, Australia) and 100 µl lysis buffer (2.5% Triton®X-114, Sigma-Aldrich, Castle Hill NSW, Australia) was added to each well and the plates were incubated for 1 h at room temperature. ALP activity was measured using a colorimetric ALP enzyme substrate (Quanti-Blue™, InvivoGen, CA, USA).

The level of IL-1α and IL-6 in the supernatant was measured using a human IL-1α enzyme-linked immunosorbent assay (ELISA) kit (Abcam Australia Pty Ltd, Melbourne, VIC, Australia), and human IL-6, mouse IL-1α and mouse IL-6 ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Immunoblotting

MG-63, MC3T3-E1, HGF-1 and NIH3T3 cells were sub-cultured in 6 well culture plates (containing the unmodified and CPP-ACP and CPP-ACFP-modified BD [0%, 0.5%, 4.0% w/w], AMTA [0%, 0.5%, 2.0% w/w] and NMTA [0%, 0.5%, 3.0% w/w]) with complete α-MEM (2×10^5 cell/well in

4 ml). After 48 h, the culture medium was replaced with 4 ml/well of the osteogenic media and the cells were incubated for 14 d. Cells cultured with osteogenic media alone (without cement) were used as control groups. The culture media were replaced every 24 h. The media for each group were collected, mixed and stored in the –80ºC freezer until use for detection of mineralisation-associated proteins (collagen type 1; Col 1, osteocalcin; OC and osteopontin; OP) by immunoblotting. To quantify the amount of the proteins, densitometry was performed using ImageJ 1.50e (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) and the results were expressed as percentages relative to the control groups.

Statistical analysis

The data were subjected to statistical analysis using SPSS ver. 11.5.0 (SPSS Inc, IL, USA). The Kolmogorov–Smirnov (K–S) test was used to test normality and the data were found to be normally distributed and therefore, parametric statistical tests were performed (one way analysis of variance followed by Tukey's test for multiple comparisons). The level of significance was set at $p \leq 0.05$.

RESULTS

Cell proliferation and cell cytotoxicity

Compared with the negative control group, all the unmodified cements increased the proliferation of MG-63 and MC3T3-E1 cells significantly, except NMTA which significantly increased the proliferation of only MC3T3-E1 cells ($p \leq 0.05$, Figure. 1). The 0.5% w/w CPP-ACP- and 4.0% w/w CPP-ACFP-modified BD, and 2.0% w/w CPP-ACFP-modified AMTA significantly reduced the proliferation of MG-63 cells compared with the unmodified cements of each material ($p \leq 0.05$). 4.0% w/w CPP-ACP- and 4.0% w/w CPP-ACFP-modified BD, 2.0% w/w CPP-ACFP-modified AMTA and 3.0% w/w CPP-ACFP-modified NMTA reduced the proliferation of MC3T3-E1 cells compared with the unmodified cements of each material significantly ($p \leq 0.05$). The proliferation of NIH3T3 cells was increased significantly by 0.5% and 4.0% w/w CPP-ACFP-modified BD, 2.0% w/w CPP-ACFP-modified AMTA and 3.0% w/w CPP-ACFP-modified NMTA compared with the unmodified cements and negative control groups ($p \leq 0.05$, Figure 2). 4.0% w/w CPP-ACFP-modified BD and 2.0% w/w CPP-ACFP-modified AMTA induced the lowest cellular proliferation of MG-63 and MC3T3-E1 cells ($p \leq 0.05$), compared with the groups of the same cements. 3.0% w/w CPP-ACFP-modified NMTA induced the lowest cellular proliferation of MC3T3-E1 cells ($p \leq 0.05$), compared with the groups of NMTA. The tested cements, with and without CPP-ACP and CPP-ACFP, had no statistically significant effect on the LDH release compared with the negative control group (Figures 3 and 4). Compared with unmodified NMTA, unmodified BD and unmodified AMTA induced higher proliferation of MG-63 cells ($p \leq 0.05$), with no statistically significant difference

between unmodified BD and unmodified AMTA. No statistically significant differences were found among unmodified BD, unmodified AMTA and unmodified NMTA in their potential to induce the proliferation of MC3T3-E1 cells.

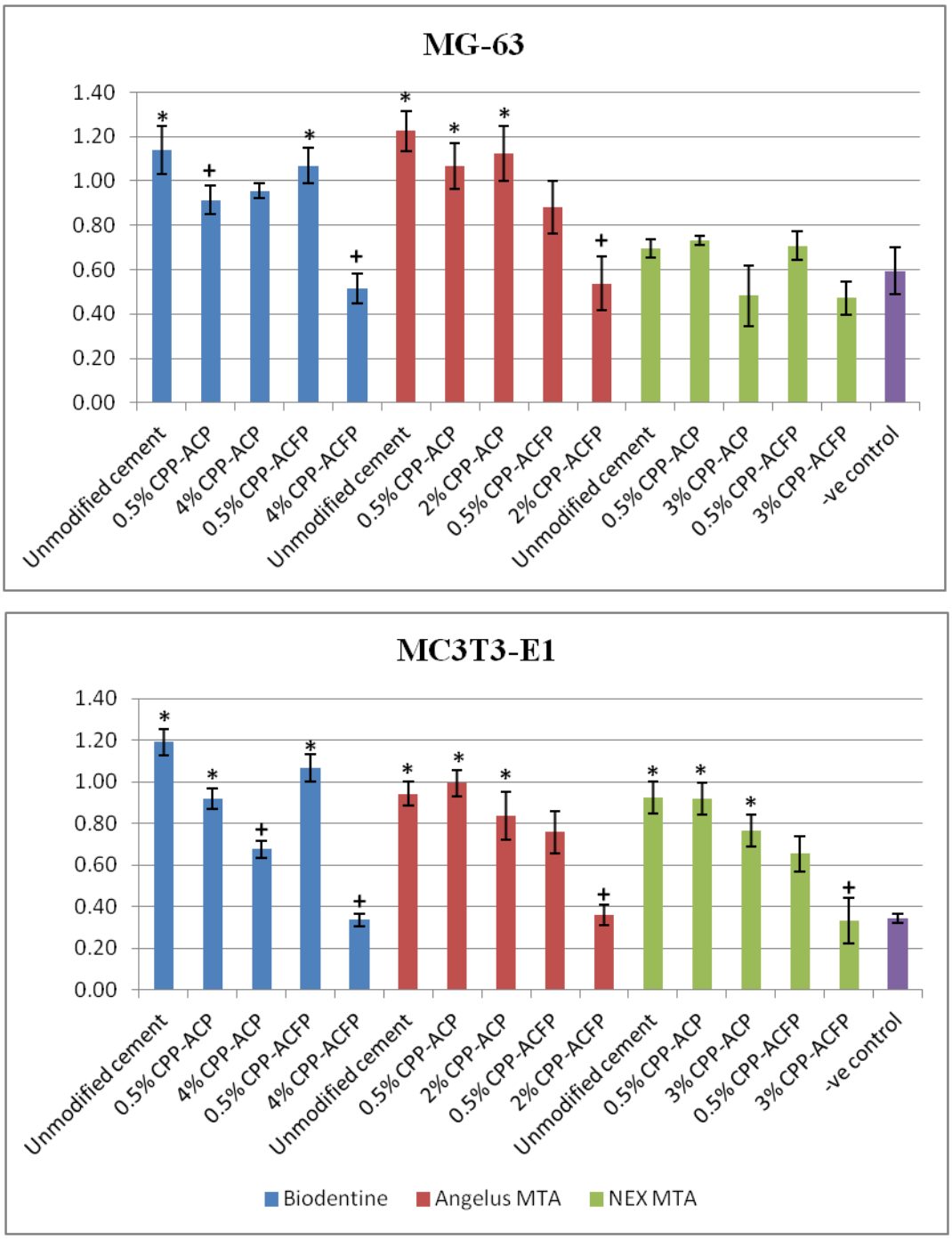


Figure 1: Proliferation (optical density at 490 nm) of osteoblast-like cell lines cultured with the unmodified and modified cements. *Denotes significant differences compared with negative control group ($P < 0.05$). +Denotes significant differences between the groups of each cement compared with the unmodified cement ($P < 0.05$).

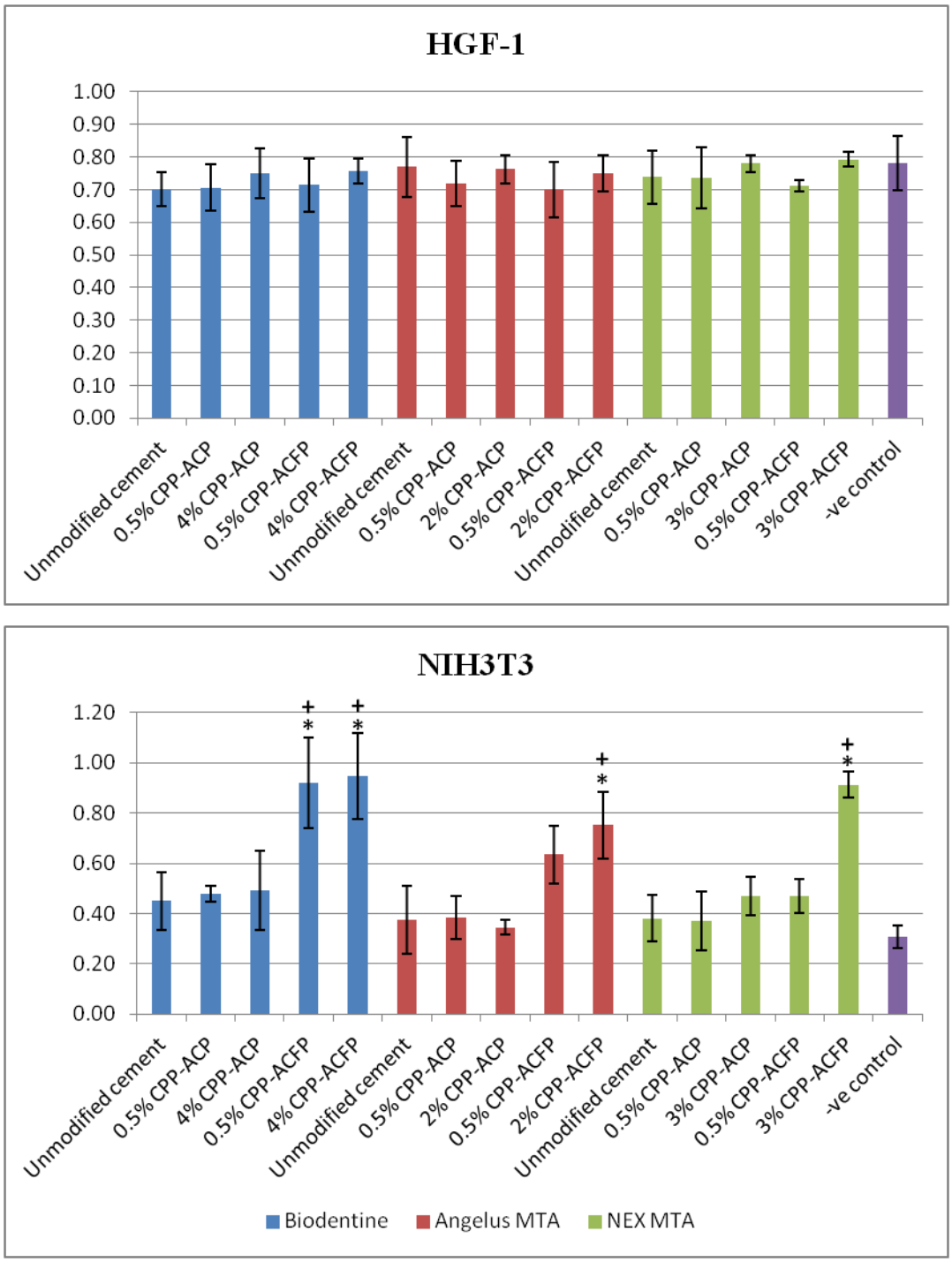


Figure 2: Proliferation (optical density at 490 nm) of fibroblast-like cell lines cultured with the unmodified and modified cements. *Denotes significant differences compared with negative control group ($P < 0.05$). +Denotes significant differences between the groups of each cement compared with the unmodified cement ($P < 0.05$).

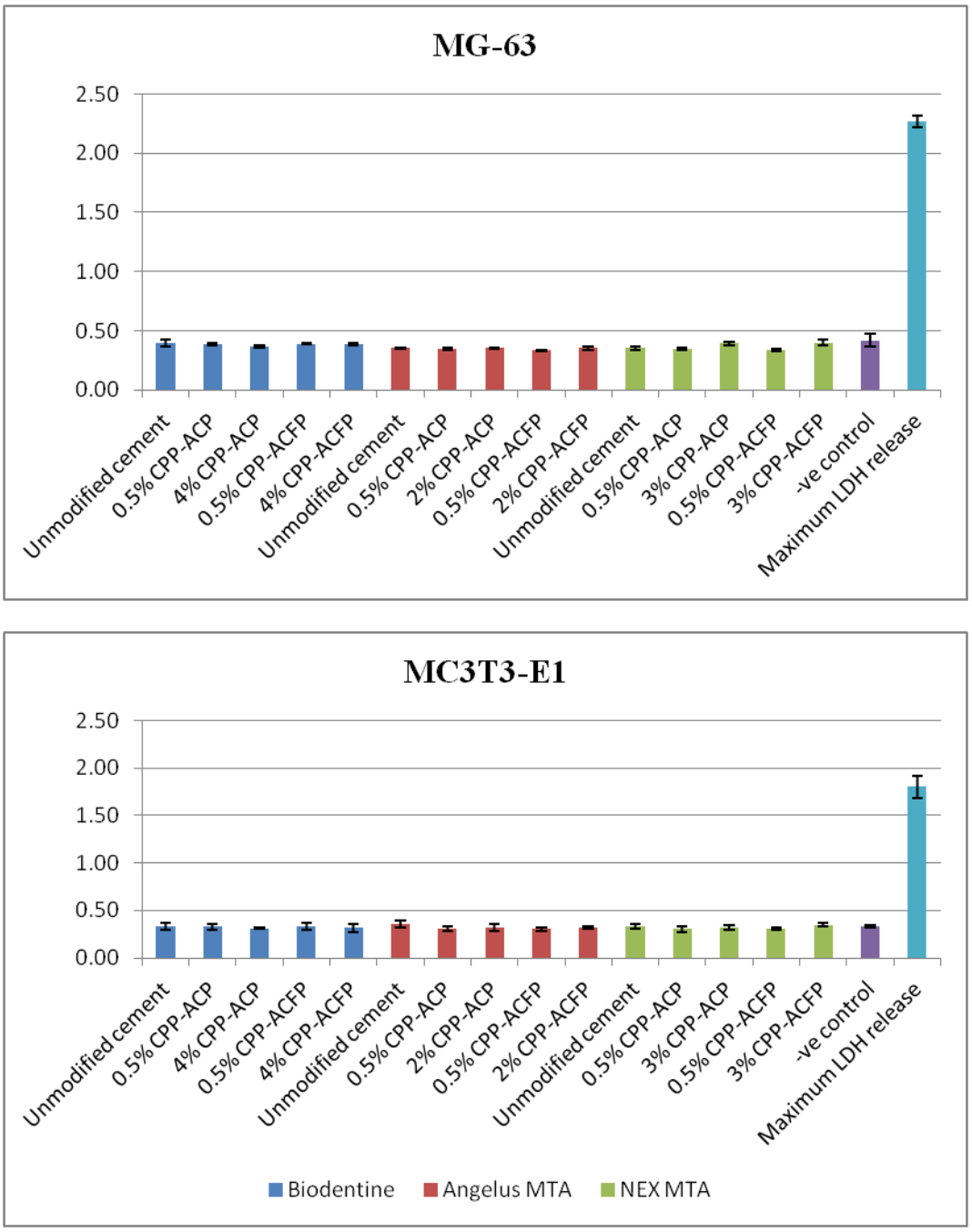


Figure 3: LDH release (optical density at 490 nm) of osteoblast-like cell lines cultured with the unmodified and modified cements. The tested cements, with and without CPP-ACP and CPP-ACFP, had no significant effect on the LDH release compared with negative control group.

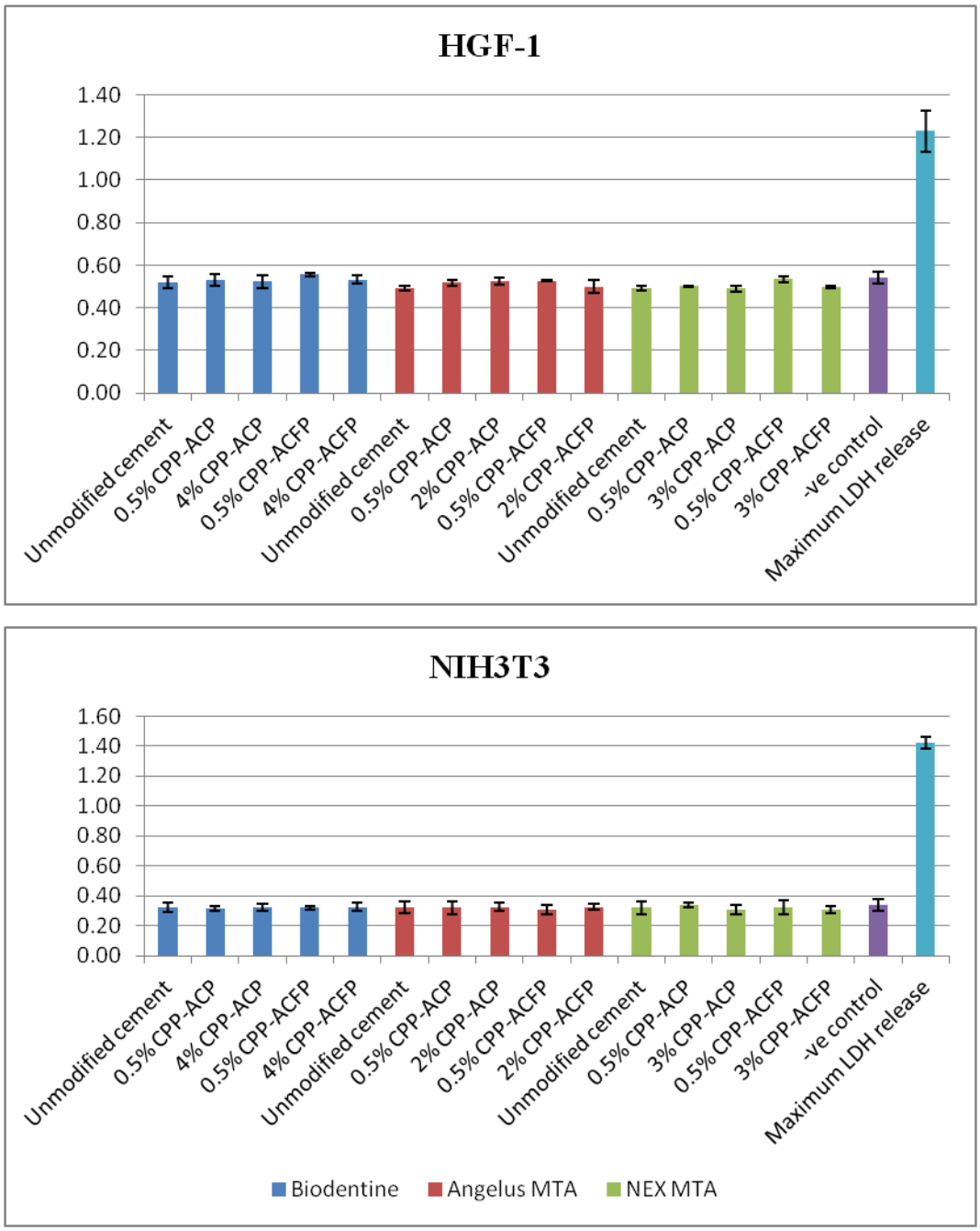
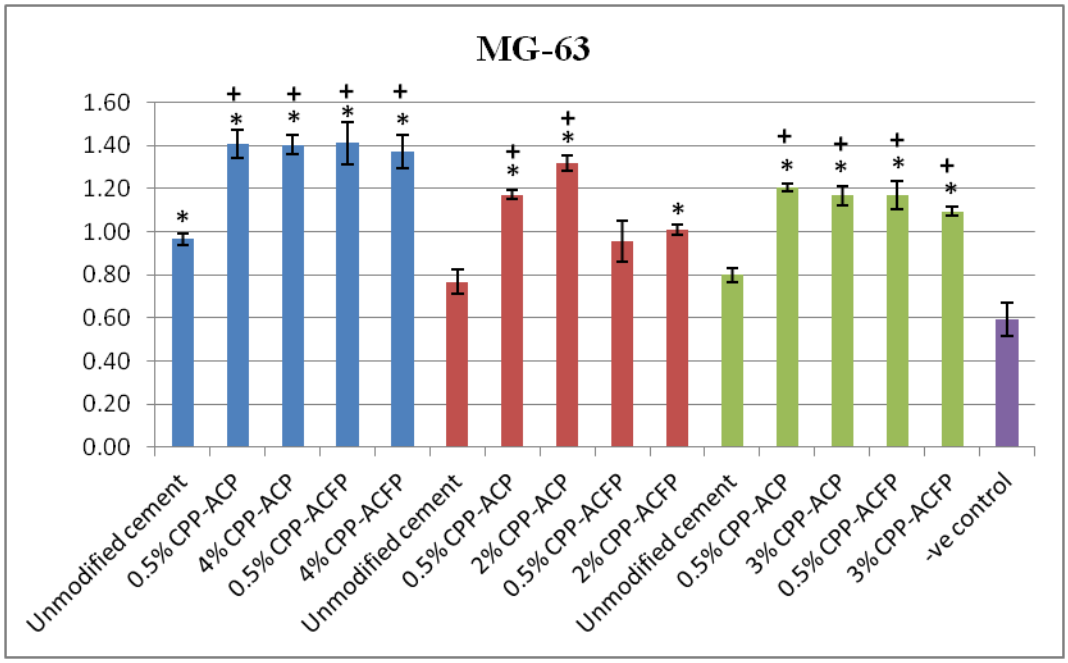


Figure 4: LDH release (optical density at 490 nm) of fibroblast-like cell lines cultured with the unmodified and modified cements. The tested cements, with and without CPP-ACP and CPP-ACFP, had no significant effect on the LDH release compared with negative control group.

Alkaline phosphatase activity

Compared with the negative control group, BD (unmodified and modified cements), 0.5% and 2.0% w/w CPP-ACP-modified AMTA, 2.0% w/w CPP-ACFP-modified AMTA, and NMTA (only the modified cements) increased the ALP activity of MG-63 cells significantly ($p \leq 0.05$, Figure 5). The addition of CPP-ACP and CPP-ACFP to BD and NMTA, and the addition of CPP-ACP to AMTA increased the ALP

activity of MG-63 cells significantly ($p \leq 0.05$) compared with the unmodified cements. The ALP activity of MC3T3-E1 cells was increased significantly by 0.5% and 4.0% w/w CPP-ACP-modified BD, 4.0% w/w CPP-ACFP-modified BD, 0.5% and 2.0% w/w CPP-ACP-modified AMTA, 3.0% w/w CPP-ACP-modified NMTA, and 0.5% w/w CPP-ACFP-modified NMTA, compared with the negative control group. The presence of 4.0% w/w CPP-ACP in BD, 0.5% and 2.0% w/w CPP-ACP in AMTA, 0.5% w/w CPP-ACFP in AMTA, and 0.5% w/w CPP-ACFP in NMTA increased the ALP activity of MC3T3-E1 cells significantly ($p \leq 0.05$) compared with the unmodified cements. No statistically significant differences were found between the unmodified cements in their potential to induce ALP activity. No statistically significant differences were found between the modified groups (of the same cement) in ALP activity, except that 4.0% w/w CPP-ACP-modified BD and 0.5% w/w CPP-ACFP-modified NMTA showed the highest ALP activity of MC3T3-E1 ($p \leq 0.05$), compared with the groups of the same cements.



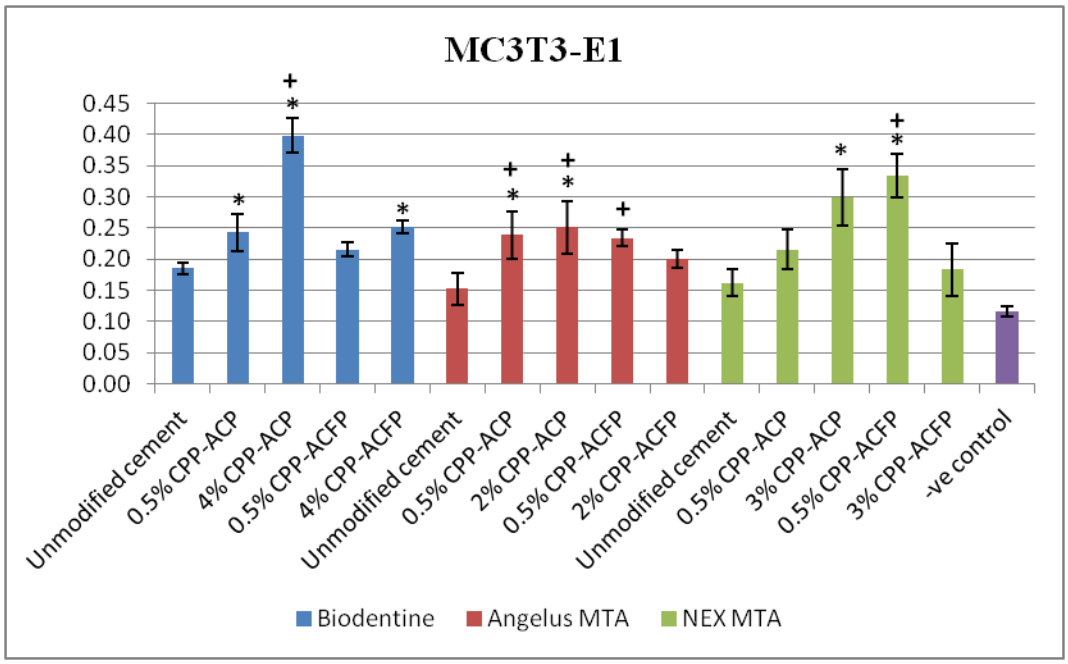


Figure 5: Alkaline phosphatase activity (optical density at 620 nm) of the MG-63 and MC3T3-E1 cell lines cultured with the unmodified and modified cements. *Denotes significant differences compared with negative control group (P < 0.05). +Denotes significant differences between the groups of each cement compared with the unmodified cement (P < 0.05).

Cytokine production

All the tested cell lines did not release IL-1α except LPS-treated cells (positive control). IL-6 was not detected in fibroblast cell lines except in the positive control groups. All the tested cements increased the release of IL-6 from MG-63 significantly compared with the negative control (Figure 6, $p \leq 0.05$). The presence of CPP-ACP and CPP-ACFP in NMTA increased the release of IL-6 from MG-63 significantly compared with unmodified NMTA ($p \leq 0.05$). MC3T3-E1 cells grown on BD (unmodified and modified groups) and 3.0% w/w CPP-ACFP-modified NMTA released significantly more IL-6 compared with the negative control ($p \leq 0.05$). 0.5% w/w CPP-ACFP-modified BD and 3.0% w/w CPP-ACFP-modified NMTA induced significantly greater IL-6 release from MC3T3-E1 compared with unmodified BD and unmodified NMTA respectively ($p \leq 0.05$). Compared with the unmodified AMTA, the unmodified BD and NMTA induced significantly greater IL-6 release from MG-63 cells ($p \leq 0.05$); no statistically significant difference was found between the unmodified BD and unmodified NMTA. 3.0% w/w CPP-ACFP-modified NMTA induced the greatest IL-6 release from MG-63 and 0.5% w/w CPP-ACFP-modified BD induced the greatest IL-6 release from MC3T3-E1 ($p \leq 0.05$), compared with the other cements.

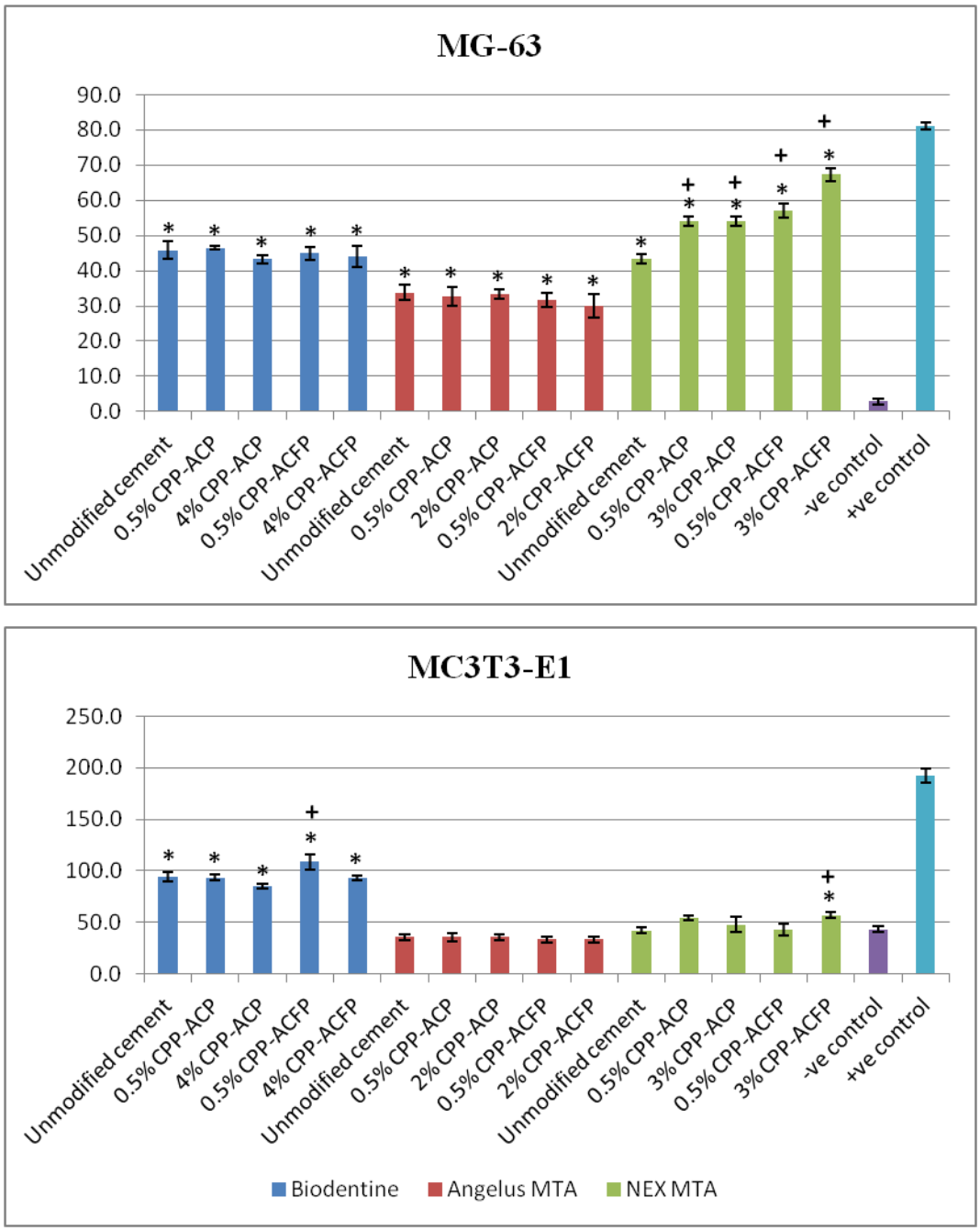


Figure 6: Concentrations of IL-6 (pg/ml) released from MG-63 and MC3T3-E1 cell lines cultured with the unmodified and modified cements. *Denotes significant differences compared with negative control group ($P < 0.05$). +Denotes significant differences between the groups of each cement compared with the unmodified cement ($P < 0.05$).

Mineralisation-associated proteins

The results of the secretion of Col 1, OP and OC are illustrated in Figures 7 and 8. There was variation among the cements in their ability to induce the secretion of mineralisation-associated proteins. In general, BD and AMTA induced the secretion of all the three proteins while NMTA mostly induced Col 1 secretion.

In comparison with unmodified BD, 4% w/w CPP-ACP-modified BD increased the secretion of Col 1 and OP from MG-63 by 128.6% and 129.7%, respectively. In comparison with unmodified BD, 0.5% w/w CPP-ACFP-modified BD increased the secretion of Col 1 and OP from MG-63 by 99.8% and 81.8%, respectively. The addition of 0.5% w/w CPP-ACP to BD increased the secretion of Col 1 from MC3T3-E1 by 26.2% in comparison with unmodified cement. The secretion of OP from MC3T3-E1 was reduced by 58.8%, 63% and 35.9% by the addition 4% w/w CPP-ACP, 0.5% w/w CPP-ACFP and 4% w/w CPP-ACFP (respectively) to BD. The presence of CPP-ACFP in BD increased the secretion of OC from MC3T3-E1 by approximately 350% in comparison with unmodified cement.

The modified AMTA induced (29.6% by 0.5% w/w CPP-ACP, 18.3% by 2% w/w CPP-ACP) higher OP secretion from MG-63 compared with unmodified cement. At 2% w/w CPP-ACFP, AMTA decreased OP secretion from MG-63 by about 30% compared with unmodified cement. The secretion of OC from MG-63 was increased by CPP-ACP-modified AMTA (about 13% - 30% in a dose-dependent manner depending on CPP-ACP concentration) and CPP-ACFP-modified AMTA (about 60%) compared with unmodified cement. CPP-ACP-modified AMTA reduced OP secretion from MC3T3-E1 by 23.3% (by 0.5% w/w CPP-ACP) and 11.8% (by 2% w/w CPP-ACP) compared with unmodified cement. While CPP-ACFP-modified AMTA increased OP secretion from MC3T3-E1 by 72.6% (by 0.5% w/w CPP-ACFP) and 52.2% (by 2% w/w CPP-ACFP) compared with unmodified cement. At 0.5% w/w CPP-ACFP, AMTA increased the secretion of OC by 82% compared with unmodified cement.

The presence of CPP-ACP or CPP-ACFP in NMTA did not increase the secretion of Col 1 nor OC from MG-63, in comparison with unmodified cement. However, the secretion of OP from MG-63 was increased by CPP-ACP-modified NMTA (15.3% with 0.5% w/w CPP-ACP and 78.7% with 3% w/w CPP-ACP) and CPP-ACFP-modified NMTA (139.6% with 0.5% w/w CPP-ACFP and 201.2% with 3% w/w CPP-ACFP) compared with unmodified cement. The addition of CPP-ACFP to NMTA reduced the secretion of OP from MC3T3-E1 by 28% (by 0.5% w/w CPP-ACFP) and 42.2% (by 3% CPP-ACFP) compared with unmodified cement.

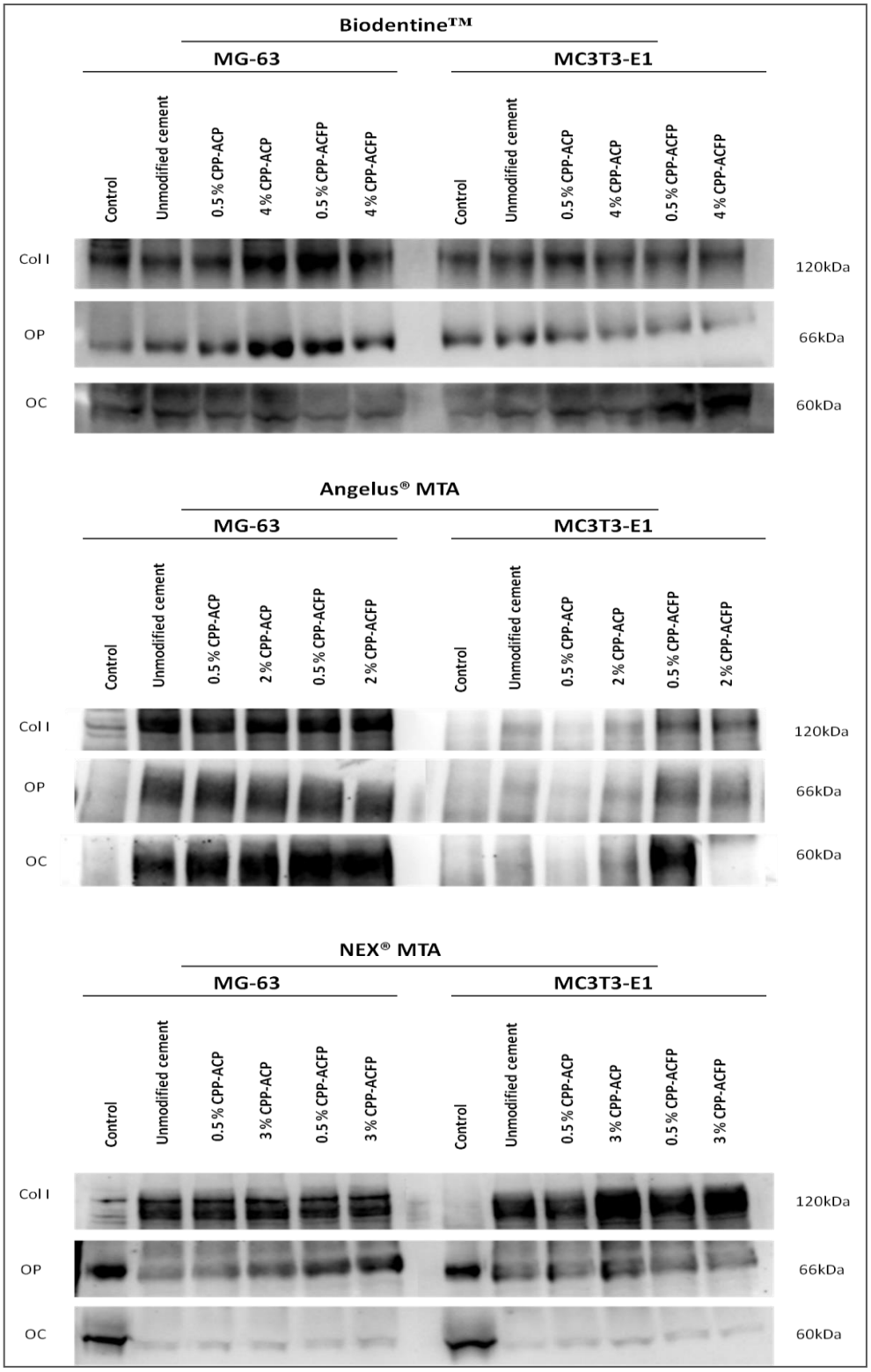


Figure 7: Secretion of mineralisation-related proteins (Collagen I; Col I, Osteopontin; OP and Osteocalcin; OC) from human and mouse osteoblast-like cells grown on the control and test cements.

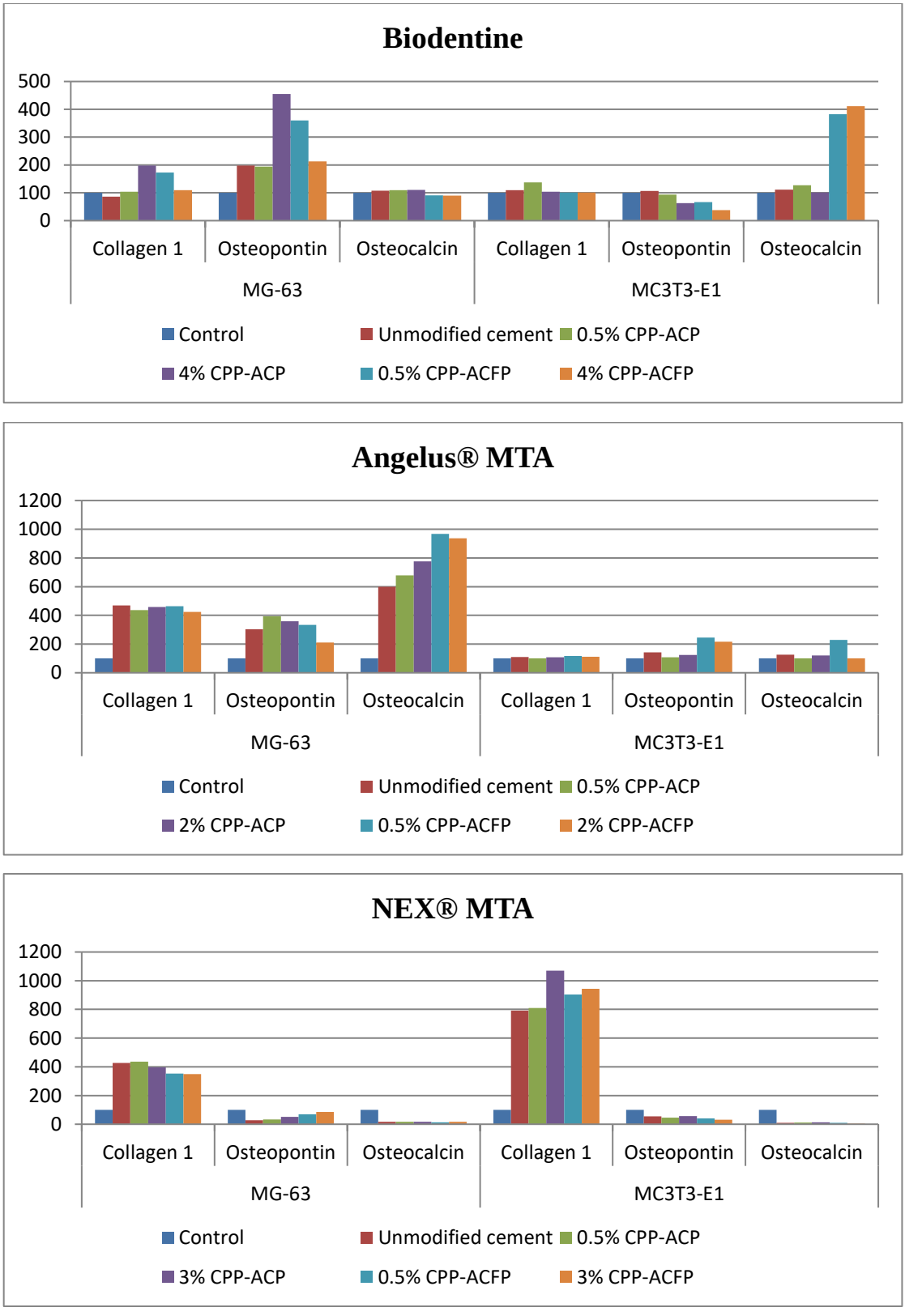


Figure 8: Percentage of mineralisation-related proteins (relative to control) secreted from human and mouse osteoblast-like cells after exposure to CPP-ACP and CPP-ACFP modified osteogenic media.

DISCUSSION

The aim of the present project was to investigate the biocompatibility and ion release of CPP-ACP- and CPP-ACFP-modified CSCs, potentially improving the bioactivity and hard tissue-forming ability of CSCs that have a wide range of applications in endodontic practice. [1] The quest for new materials, by improving the available cements or producing new formulations, is a continuous process that aims to enhance the outcome of the clinical treatment modalities routinely performed in dentistry.

In general, the proliferation of osteoblast-like cells grown on the cements (especially the unmodified cements) was higher compared with the negative control. This could be due to the cements acting as better scaffolds in comparison with the plastic of the culture plates. It has been reported that human dental pulp cells seeded on MTA showed better growth patterns as they appeared to be flat and exhibited well-defined cytoplasmic extensions compared with the negative control. [16] A previous study confirmed the biocompatibility of MTA, and that the proliferation of a pluripotent-mesenchymal cell line treated with MTA extract was significantly increased compared with untreated cells. [17] Another study showed that MG-63 cells grown on the surface of MTA had significantly higher proliferation compared with the control. [6]

On the other hand, the present findings showed that the addition of CPP-ACP or CPP-ACFP to CSCs reduced the proliferation of osteoblast-like cells. The down-regulation of proliferation of osteoblast-like cells could be a sign of the osteoblastic differentiation induced by the incorporated CPP-ACP and CPP-ACFP, as the cells undergo growth changes during the differentiation pathway toward final maturation. [18]

The tested cements had no effect on the proliferation of HGF-1 cells, and the proliferation of NIH3T3 cells was increased only by CPP-ACFP-modified cements (especially the high concentrations). All the tested cements did not reveal any cytotoxic effect as they did not increase the LDH release in comparison with the negative control. The present findings of the proliferation and LDH release indicate that the tested cements are biocompatible, and they could serve as suitable scaffolds for the proliferation of osteoblast and fibroblast cells.

The increase in the ALP activity of osteoblast-like cells induced by the tested cements could be due to the osteoblastic differentiation promoted by these cements. ALP enzyme is an osteoblastic differentiation marker and it has a significant role in hard tissue formation [19].

The tested cements did not trigger an inflammatory response as there was no IL-1α (sterile inflammation cytokine) detected.[20] IL-6 has an important role in osteoblastic differentiation by enhancing ALP activity.[21] Ca^{2+} released from calcium-enriched CPP was reported to

increase IL-6 release [22], which may explain the increase in IL-6 release reported in the present study which might have been part of the process of osteoblastic differentiation induced by Ca^{2+} released from the tested cements. The findings of the present study regarding IL-6 is in agreement with previous researchers reporting no IL-1α release along with an expression of IL-6 from MG-63 and MC3T3-E1 cells grown in intimate contact with MTA. [23]

In general, the present findings revealed that the tested cements (especially BD, AMTA and CPP-ACFP-modified cements) induced the secretion of mineralisation-associated proteins (especially Col 1 and OC). Previous reports also indicated that growing MG-63 on MTA resulted in increased IL-6, Col 1, OC and ALP expression compared with control groups. [6] MC3T3-E1 cells grown on CSCs were reported to have a significantly increased expression of Col 1, OC and OP genes compared with the control group.[24] A previous study showed that MTA induced odontoblastic differentiation of human dental pulp cells, and this was evidenced by expression of odontogenic-related proteins and formation of mineralised nodules. [16] The variations amongst the tested cements in their potential to induce secretion of mineralisation-associated proteins could be attributed to the differences in their ability to release Ca^{2+} [25].

The present findings showed that the addition of CPP-ACP and CPP-ACFP to CSCs promoted osteoblastic differentiation, possibly by increasing Ca^{2+} and P*i* release. Ca^{2+} released from MTA has been reported to convert the differentiation pathway of the pluripotent-mesenchymal cell line into osteoblasts and/or chondroblasts. [17] Besides being a necessary component for hydroxyapatite crystal formation, P*i* is believed to be an important signalling molecule in osteoblastic differentiation. [26] The ability of endodontic cements to release Ca^{2+} and P*i*, and to form apatite-like crystals may induce odontoblastic differentiation and reparative dentine formation. [27] However, these findings require further studies (like animal studies) to test the efficiency of using CPP-ACP-modified and CPP-ACFP-modified CSCs in vital pulp therapy and osteo-genesis.

CONCLUSION

The tested cements are biocompatible, and they could serve as suitable scaffold for the proliferation of osteoblast and fibroblast cells. The addition of CPP-ACP and CPP-ACFP to CSCs improved the cements' potential to induce osteoblastic differentiation. This improvement may enhance the outcome of the therapeutic dental procedures that involve the use of CSCs such as direct pulp capping, root perforation repair and apexification. Further studies (especially animal studies) are recommended to assess the biological behaviour of the modified cements.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication and/or funding of this manuscript.

REFERENCES

1. Parirokh M, Torabinejad M. Mineral trioxide aggregate: a comprehensive literature review-Part III: clinical applications, drawbacks, and mechanism of action. Journal of Endodontics. 2010;36:400-13.

2. Mohammadi Z, Dummer PMH. Properties and applications of calcium hydroxide in endodontics and dental traumatology. International Endodontic Journal. 2011;44: 697-730.

3. Sun J, Wei L, Liu X. Influence of ionic dissolution products of dicalcium silicate coating on osteoblastic proliferation, differentiation and gene expression. Acta Biomaterialia. 2009;5:1284-93.

4. Sangwan P, Sangwan A, Duhan J, Rohilla A. Tertiary dentinogenesis with calcium hydroxide: a review of proposed mechanisms. International Endodontic Journal. 2013;46:3-19.

5. Estrela C, Sydney GB, Bammann LL, Felippe Junior O. Mechanism of action of calcium and hydroxyl ions of calcium hydroxide on tissue and bacteria. Brazilian Dental Journal. 1995;6:85-90.

6. Chen CL, Huang TH, Ding SJ. Comparison of calcium silicate cement and mineral trioxide aggregate biologic effects and bone markers expression in MG63 cells. Journal of Endodontics. 2009;35:682-5.

7. Sarkar NK, Caicedo R, Ritwik P, Moiseyeva R, Kawashima I. Physicochemical basis of the biologic properties of mineral trioxide aggregate. Journal of Endodontics. 2005;31:97-100.

8. Gandolfi MG, Siboni F, Polimeni A, Bossu M, Riccitiello F, Rengo S, et al. In vitro screening of the apatite-forming ability, biointeractivity and physical properties of a tricalcium silicate material for endodontics and restorative dentistry. Dentistry Journal. 2013;1:41-60.

9. Reynolds EC. Anticariogenic complexes of amorphous calcium phosphate stabilized by casein phosphopeptides: a review. Special Care in Dentistry. 1998;18:8-16.

10. Nongonierma AB, Fitzgerald RJ. Biofunctional properties of caseinophosphopeptides in the oral cavity: a review. Caries Research. 2012;46:234-67.

11. Cochrane NJ, Saranathan S, Cai F, Cross KJ, Reynolds EC. Enamel subsurface lesion remineralisation with casein phosphopeptide stabilised solutions of calcium, phosphate and fluoride. Caries Research. 2008;42:88-97.

12. Donida BM, Mrak E, Gravaghi C, Villa I, Cosentino S, Zacchi E, et al. Casein phosphopeptides promote calcium uptake and modulate the differentiation pathway in human primary osteoblast-like cells. Peptides. 2009;30:2233-41.

13. Tulipano G, Bulgari O, Chessa S, Nardone A, Cocchi D, Caroli A. Direct effects of casein phosphopeptides on growth and differentiation of in vitro cultured osteoblastic cells (MC3T3-E1). Regulatory Peptides. 2010;160:168-74.

14. Gandolfi MG, Taddei P, Tinti A, Dorigo De Stefano E, Rossi PL, Prati C. Kinetics of apatite formation on a calciumsilicate cement for root-end filling during ageing in physiological-like phosphate solutions. Clinical Oral Investigations. 2010;14:659-68.

15. Dawood AE, Manton DJ, Parashos P, Wong RH, Singleton W, Holden JA, et al. Biocompatibility and Osteogenic/Calcification Potential of Casein Phosphopeptide-amorphous Calcium Phosphate Fluoride. J Endod. 2018;44:452-7.

16. Lee JB, Park SJ, Kim HH, Kwon YS, Lee KW, Min KS. Physical properties and biological/odontogenic effects of an experimentally developed fast-setting α-tricalcium phosphate-based pulp capping material. BMC Oral Health. 2014;14:87.

17. Matsumoto S, Hayashi M, Suzuki Y, Suzuki N, Maeno M, Ogiso B. Calcium ions released from mineral trioxide aggregate convert the differentiation pathway of C2C12 cells into osteoblast lineage. Journal of Endodontics. 2013;39:68-75.

18. Lian JB, Stein GS. The developmental stages of osteoblast growth and differentiation exhibit selective responses of genes to growth factors (TGF beta 1) and hormones (vitamin D and glucocorticoids). The Journal of Oral Implantology. 1993;19:95-105.

19. Golub EE, Boesze-Battaglia K. The role of alkaline phosphatase in mineralization. Current Opinion in Orthopaedics. 2007;18:444-8.

20. Lukens JR, Gross JM, Kanneganti TD. IL-1 family cytokines trigger sterile inflammatory disease. Frontiers in Immunology. 2012;3:315.

21. Erices A, Conget P, Rojas C, Minguell JJ. Gp130 activation by soluble interleukin-6 receptor/interleukin-6 enhances osteoblastic differentiation of human bone marrow-derived mesenchymal stem cells. Experimental Cell Research. 2002;280:24-32.

22. Kitts DD, Nakamura S. Calcium-enriched casein phosphopeptide stimulates release of IL-6 cytokine in human epithelial

intestinal cell line. Journal of Dairy Research. 2006;73:44-8.

23. Deller-Quinn M, Perinpanayagam H. Osteoblast expression of cytokines is altered on MTA surfaces. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2009;108:302-7.

24. Yuan Z, Peng B, Jiang H, Bian Z, Yan P. Effect of bioaggregate on mineral-associated gene expression in osteoblast cells. Journal of Endodontics. 2010;36:1145-8.

25. Dawood AE, Manton DJ, Parashos P, Wong RH, Palamara JE, Stanton DP, et al. The physical properties and ion release of CPP-ACP-modified calcium silicate-based cements. Australian Dental Journal. 2015;60:434-44.

26. Beck G. Inorganic phosphate as a signaling molecule in osteoblast differentiation. Journal of Cellular Biochemistry. 2003;90:234-43.

27. Wang X, Suzawa T, Miyauchi T, Zhao B, Yasuhara R, Anada T, et al. Synthetic octacalcium phosphate-enhanced reparative dentine formation via induction of odontoblast differentiation. Journal of Tissue Engineering and Regenerative Medicine. 2013;9:1310-20.