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Osteogenic Response of Saos-2 Cells to Calcium Silicate-based versus Hydroxyapatite-based Cements: A Comparative Study

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Abstract

Introduction: Bioceramic materials are integral to regenerative endodontics, yet comparative data on their osteogenic potential remain limited. This study compared the osteogenic potential of four bioceramics: hydroxyapatite (HA), a nano-hydroxyapatite/chitosan composite (n-HA/CSS), Angelus mineral trioxide aggregate (MTA), and calcium-enriched mixture (CEM) cement. **Materials and Methods:** Human osteoblast-like Saos-2 cells were exposed to materials *via* direct contact and extract models. Cell viability and proliferation were assessed using the MTT assay, attachment and morphology were examined by scanning electron microscopy (SEM), and osteogenic differentiation was evaluated by real-time PCR for alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OC) at 3 and 6 days. **Results:** All materials were non-cytotoxic. In direct culture, CEM significantly enhanced cell proliferation by day 3 (~3-fold, $P<0.001$). SEM revealed superior cell attachment and extracellular matrix secretion on n-HA/CS and CEM surfaces. Gene expression analysis demonstrated that CEM robustly upregulated ALP (~12-fold *vs.* control, $P<0.001$), a level 6-fold higher than MTA ($P<0.05$). Furthermore, both CEM and n-HA/CS promoted pronounced expression of the late markers BSP (~5-fold, $P<0.001$) and OC (10-fold and 12-fold, $P<0.001$ and $P<0.001$, respectively). MTA consistently elicited the lowest osteogenic response. **Conclusions:** CEM and the n-HA/CS were identified as the strongest promoters of osteoblast proliferation and differentiation, indicating their superior bioactivity over MTA and HA and their potential for endodontic and surgical hard tissue regeneration.

Keywords: Biocompatible Materials; Calcium-enriched Mixture Cement; Calcium Silicate Materials; Hydroxyapatite; Osteogenesis

Introduction

Bioceramic materials are widely used in endodontics because of their ability to interact with dental and periapical tissues through apical foramina, root perforations, or root-end fillings, thereby promoting healing/regeneration [1]. The success of pulp and periapical therapies largely depends on the capacity of restorative materials to be biocompatible, provide a durable seal, and stimulate hard tissue formation [2, 3].

Calcium silicate–based materials represent the most widely investigated class of endodontic biomaterials. Among them, mineral trioxide aggregate (MTA), first introduced in the early 1990s, has been extensively studied for root-end filling, pulp covering, and perforation repair [4]. MTA consists primarily of tricalcium silicate, dicalcium silicate, tricalcium aluminate, and bismuth oxide, and its bioactivity is attributed to hydration reactions that release calcium hydroxide, promoting hydroxyapatite deposition at the material–tissue interface [5, 6].

These features confer excellent sealing ability, high biocompatibility, and the capacity to stimulate cementogenesis and osteogenesis, which make MTA clinically valuable. However, significant drawbacks limit its routine use, including an extended setting time, difficult handling properties, potential tooth discoloration, relatively high cost, and inconsistent antimicrobial efficacy [4].

To address these limitations, calcium-enriched mixture (CEM) cement was introduced as a second-generation calcium silicate–based biomaterial. Calcium-enriched Mixture (CEM) is a water-based, tooth-colored cement composed of various calcium compounds with physicochemical and handling properties distinct from MTA [3]. Compared with MTA, CEM demonstrates shorter setting time, enhanced flowability, reduced film thickness, and lower microleakage, while preserving favorable sealing ability and bioactivity [3]. Importantly, CEM exhibits antimicrobial properties comparable to calcium hydroxide [7, 8] and has been shown to promote hard tissue induction [9], making it an attractive alternative to other biomaterials in regenerative endodontic procedures.

In parallel, calcium phosphate–based biomaterials such as hydroxyapatite (HA) have been investigated as alternatives for dental and orthopedic applications [10]. HA is structurally analogous to the mineral phase of bone, offering biocompatibility, osteo-conductivity, and osteo-inductivity [11]. Advances in nanotechnology have led to the development of nano-hydroxyapatite (n-HA), which more closely resembles natural bone apatite in particle size and surface properties. n-HA exhibits improved mechanical strength, higher bioactivity, and greater capacity for ionic exchange compared with conventional HA [12]. Furthermore, combining n-HA with natural polymers such as chitosan enhances bioactivity, biodegradability, and antimicrobial potential, thereby improving bone regeneration [13].

Although the physicochemical and bioactivity characteristics of MTA, CEM, HA, and n-HA cements have been extensively studied, there remains a lack of systematic comparative studies evaluating their biological effects, particularly concerning osteogenic potential. Therefore, this *in vitro* study aimed to assess the cytocompatibility, proliferation, and differentiation of human osteoblast-like Saos-2 cells (Saos-2) exposed to these biomaterials. Specifically, we examined cell viability, ultrastructural morphology, and the expression of osteogenic markers [alkaline phosphatase (ALP), bone sialoprotein (BSP), and osteocalcin (OC)] to provide a comparative evaluation of their regenerative potential.

Materials and Methods

Cement preparation and sterilization

According to the manufacturers' instructions, Angelus MTA (Angelus, Londrina, Brazil) and CEM cement (BioniqueDent, Tehran, Iran) were prepared by mixing powder and liquid at predefined powder-to-liquid ratios. HA cement was prepared by combining tetracalcium phosphate and dicalcium phosphate dihydrate as the solid phases with sodium dihydrogen phosphate (NaH_2PO_4) as the liquid phase. The n-HA/Chitosan (n-HA/CS) cement consisted of a solid phase (chitosan, nano-HA, $CaCO_3$, and CaO) and a liquid phase (acetic acid, citric acid, NaH_2PO_4, $CaCl_2$, and distilled water). For HA and n-HA/CS preparation, the powder-to-liquid ratios were 3:1 and 0.83:1 (g/mL), respectively [14].

All cements were mixed under aseptic conditions and placed into cylindrical molds (10 mm × 3 mm). The samples were incubated at 37 ˚C in 95% humidity for 24 h to allow complete setting. Following incubation, both sides of each sample were disinfected by ultraviolet (UV) irradiation for 20 min before biological testing.

(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

A human osteosarcoma cell line (Saos-2; NCBI code: C453) was obtained from the Pasteur Institute cell bank (Pasteur Institute of Iran, Tehran, Iran). Despite their malignant origin, Saos-2 cells are widely used in bone and dental research because they reproduce key functions and phenotypic characteristics of mature human osteoblasts, making them a reliable permanent model for osteoblast-like cells [15, 16]. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Paisley, Scotland, UK) supplemented with 10% fetal bovine serum (FBS; Gibco, UK) and 1% penicillin–streptomycin (Gibco, UK), hereafter referred to as complete or regular medium (RM). Cultures were maintained at 37 ˚C in a humidified atmosphere with 5% CO_2 and 95% humidity. Cells in the logarithmic growth phase were used for MTT and quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays.

To assess cytotoxicity, viability, and proliferation in the presence of the test cements, two methods were employed: direct contact and indirect contact. In the direct method, 10,000 cells were seeded onto the surface of UV-sterilized cement discs (24-well plates) that had been pre-incubated in medium for 24 h (to hydrate and exclude contamination). Viability was assessed at 1, 3, and 6 days using the MTT assay, following ISO 10993-5:2009 guidelines. In the indirect method, UV-sterilized cement discs were incubated in culture medium supplemented with FBS (1 mL/cm² of specimen surface) for 24 h at 37 ˚C, 5% CO_2, and 95% humidity. The resulting extract solutions were filtered through sterile 0.22 µm filters and added, without dilution, to pre-seeded Saos-2 cells (10,000 cells/well in 96-well plates). Cell viability was

measured at 1 and 2 days, following ISO 10993-12:2021 standards. For controls, RM served as the negative control (NC; 100% viability), and distilled water as the positive cytotoxicity control (<10% viability) [17], and osteogenic medium (OM, containing vitamin C, dexamethasone, and glycerophosphate) as the positive control for differentiation.

For the MTT assay, culture medium was replaced with DMEM (without FBS or antibiotics) containing 10% MTT dye (5 mg/mL stock, Sigma-Aldrich Chemie, Taufkirchen, Germany) at final volumes of 100 µL (96-well) or 1000 µL (24-well). Plates were incubated for 3 h at 37 ˚C. After crystal formation, the medium was removed and replaced with dimethyl sulfoxide (Sigma-Aldrich, Germany) to dissolve formazan. Aliquots of 100 µL were transferred to 96-well plates, and absorbance was measured using a microplate reader (Anthous 2020; Anthos Labtec Instruments, Wals-Siezenheim, Austria) at 570 and 620 nm. In indirect assays, the percentage of viable cells was calculated as (OD of test group/OD of RM control) × 100. According to ISO 10993-5 (2009), viability <70% relative to controls was considered cytotoxic. In both direct and indirect contact experiments, proliferation was determined from OD changes across time points [17, 18].

Scanning electron microscopy (SEM)

Saos-2 cells (7×10^3 per sample) were seeded onto the surface of the cement specimens and incubated for 24 h at 37 ˚C in a humidified atmosphere containing 5% CO_2 and 95% humidity. Following incubation, the specimens were gently rinsed with phosphate-buffered saline to remove non-adherent cells. The attached cells were fixed in 2.5% glutaraldehyde solution (Sigma-Aldrich, Germany) for 1h at room temperature. Samples were then dehydrated in a graded ethanol series (30%, 50%, 70%, 80%, 90%, and 100%), with each step lasting 10 min. After dehydration, the specimens were air-dried for 24 h, sputter-coated with a thin layer of gold, and subsequently examined by SEM.

Real-time PCR

The qRT-PCR was performed to evaluate the expression of genes involved in osteogenic differentiation, including ALP (early marker), BSP (mid-to-late marker), and OC (late marker). Saos-2 cells (7×10^3 per well) were cultured and treated with extract solutions of the tested cements for 3 and 6 days. RM and OM were used as negative (NC) and positive (PC) controls for osteogenic differentiation, respectively.

Total RNA was extracted using a commercial kit (Qiagen, Bodenseeallee, Stockach, Germany), and complementary DNA (cDNA) was synthesized according to the manufacturer's instructions. Relative mRNA expression was quantified using the SYBR Green qPCR kit (Applied Biosystems, Foster City, CA, USA). Ribosomal protein L13a (RPL13a), one of the most stable reference genes during osteogenic, adipogenic, and chondrogenic differentiation [19], was used as the endogenous control. Primer sequences for all target and reference genes are provided in Table 1.

Gene expression data were analyzed using the comparative Ct method (ΔΔCt). For each sample, mean cycle threshold (Ct) values were normalized to RPL13a, and fold changes in expression of ALP, BSP, and OC were calculated relative to RM control (NC) samples.

Statistical analysis

All data were analyzed using GraphPad Prism software (Version 9, GraphPad Software, San Diego, CA, USA). Results are presented as mean±standard deviation (SD) from three independent experiments, each performed in triplicate. Differences between groups were assessed using two-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. A *P*-value of <0.05 was considered statistically significant.

Table 1. Primer sequences used for quantitative real-time PCR analysis of alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin (OC), and ribosomal protein L13a (RPL13a, reference gene)

Primer Name	Sequences (5'->3')	Annealing Time	Product Length	PCR efficiency (%) Regression coefficient (R2)
ALP **(Early marker)**	F: ATTTCTCTTGGGCAGGCAGAGAGT R: ATCCAGAATGTTCCACGGAGGCTT	60 ˚C	118 bp	103.89 0.999
BSP **(Mid-late marker)**	F: GCAGTAGTGACTCATCCGAAGAA R: GCCTCAGAGTCTTCATCTTCATTC	60 ˚C	121 bp	105.68 0.998
OC **(Late marker)**	F: CAGCGAGGTAGTGAAGAGAC R: TGAAAGCCGATGTGGTCAG	60 ˚C	144 bp	103.89 0.996
RPL 13a (Reference gene)	F: CATAGGAAGCTGGGAGCAAG R: GCCCTCCAATCAGTCTTCTG	60 ˚C	157 bp	110.77 0.999

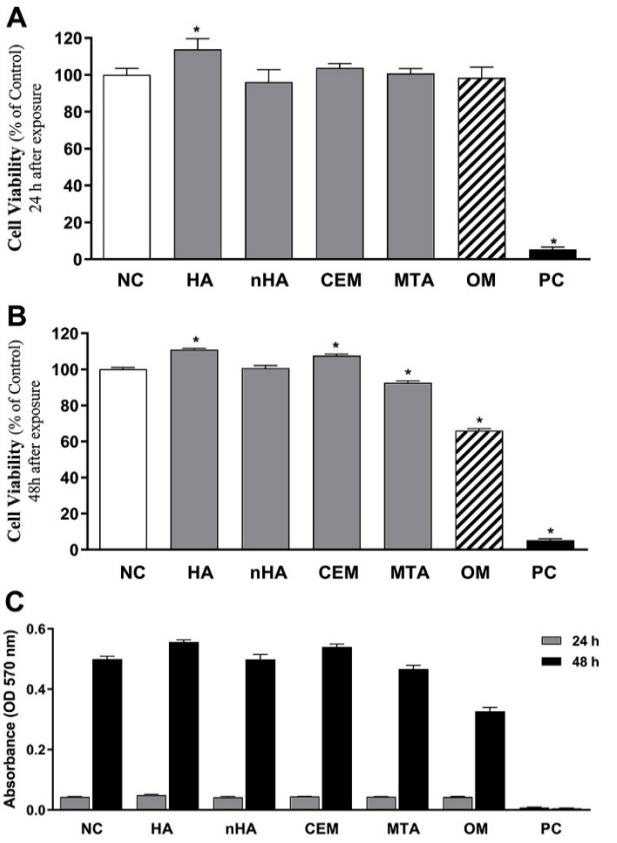

Figure 1. Viability of Saos-2 cells following indirect exposure to cement extracts. *A)* Cell viability after 24 h; *B)* cell viability after 48 h, and *C)* proliferation dynamics shown as absorbance values (OD at 570 nm). Test groups: hydroxyapatite (HA), nano-hydroxyapatite (n-HA), calcium-enriched mixture (CEM), and mineral trioxide aggregate (MTA). Controls: NC (negative control, 100% viability), PC (positive control, cytotoxic), and OM (osteogenic medium). Data are presented as mean±SD from three independent experiments (n=3), P<0.05. Asterisks above columns indicate significance *versus* NC

Results

MTT assay

Indirect exposure: As shown in Fig. 1A, after 24 h exposure to cement extracts, cells in the HA group exhibited a significant increase in viability (13.8%) compared with the control group (RM, set at 100% viability; P=0.024). No statistically significant differences were observed in the n-HA/CS, CEM, MTA, or OM groups compared with RM (P=0.775, 0.773, 0.999, and 0.995, respectively). However, pairwise comparisons revealed significant differences between HA and n-HA/CS, as well as between CEM and MTA. Importantly, no cytotoxic effects were detected in any group, as viability values remained above the 70% threshold. At 48 h (Fig. 1B), cell viability increased by 11.1% in the HA group

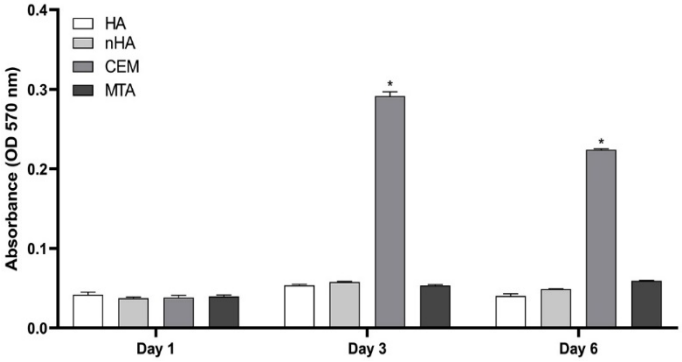

Figure 2. Proliferation of Saos-2 cells in direct contact with different cements (HA, n-HA/CS, CEM, MTA) for 1, 3, and 6 days. Proliferation is expressed as absorbance values (OD at 570 nm). Data are shown as mean±SD (n=3). P<0.05. Asterisks above columns indicate significant differences relative to other groups

(P<0.001) and by 7.7% in the CEM group (P=0.014) compared with RM. In contrast, viability decreased significantly in the MTA (−10.5%; P<0.001) groups relative to RM. None of the experimental groups fell below 70% viability, indicating the absence of cytotoxicity. The reduced viability in OM is likely attributable to suppressed proliferation and initiation of osteogenic differentiation rather than cytotoxicity.

Cell proliferation dynamics are illustrated in Fig. 1C. Across all groups, cell numbers were significantly higher at 48 h compared with 24 h. Proliferation rates in the HA and CEM groups exceeded those of RM and other experimental groups, whereas both MTA groups and OM showed comparatively reduced proliferation.

Direct exposure: As illustrated in Fig. 2, no significant differences in OD values were detected between days 1, 3, and 6 in any group except CEM. In the CEM group, a marked increase in proliferation was observed on day 3 compared with day 1 (~3-fold; P<0.001). Although OD values declined on day 6 relative to day 3, they remained significantly higher than in all other groups at the same time point (~2-fold; P<0.001)

SEM

Fig. 3 presents SEM micrographs of Saos-2 cells cultured on the surface of the tested cements after 3 days. In all groups, cells adhered well to the cement surfaces, exhibiting typical osteoblast-like morphology with extracellular matrix (ECM) deposition and cytoplasmic extensions (filopodia) connecting to the substrate and neighboring cells.

Cells cultured on n-HA/CS demonstrated particularly strong anchorage, abundant ECM secretion, and prominent intercellular contacts, suggesting accelerated progression toward differentiation compared with other groups. Similarly, CEM surfaces supported extensive cell spreading, dense matrix

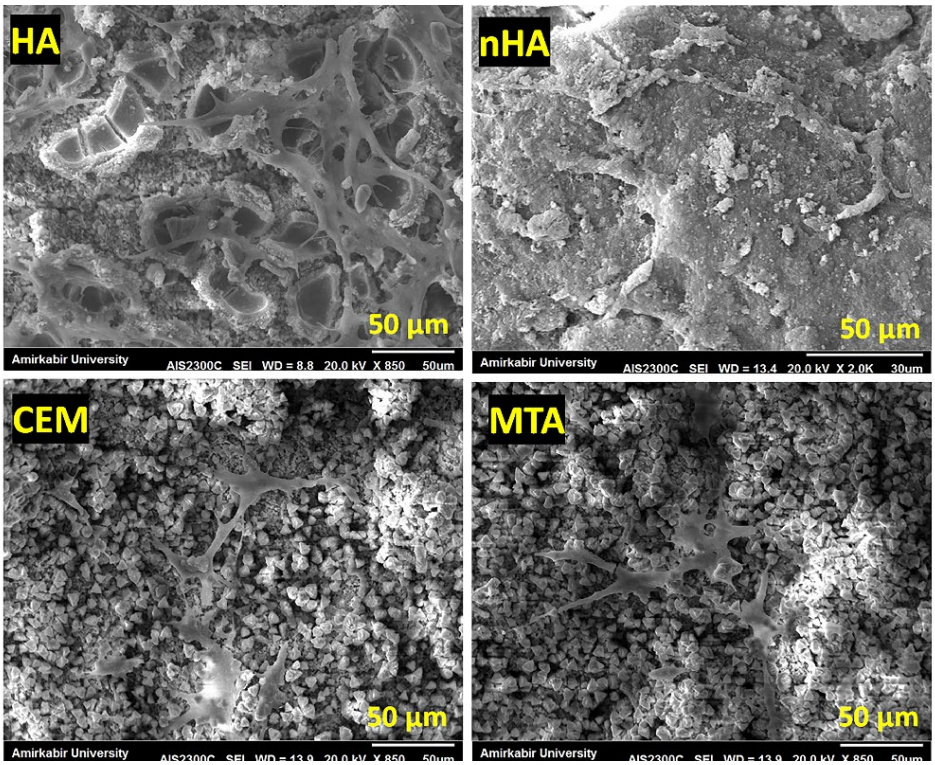

Figure 3. SEM micrographs of Saos-2 cells cultured on the surfaces of CEM, MTA, HA, and n-HA/CS cements after 3 days. Cells show attachment, extracellular matrix secretion, and cytoplasmic extensions

deposition, and multiple filopodial extensions. By contrast, cells on MTA and HA surfaces displayed comparatively less coverage and fewer extensions. Overall, qualitative analysis indicated more favorable cell–material interactions in the n-HA/CS and CEM groups relative to MTA and HA.

Gene expression

ALP expression: Fig. 4A shows ALP gene expression in Saos-2 cells after exposure to cement extracts. Expression in the RM group (NC: negative control) was set to 1. By day 6, ALP was significantly upregulated in all groups except MTA, indicating induction of early differentiation. In the CEM group, ALP expression reached ~12-fold relative to NC, 1.5-fold higher than the PC, 6-fold higher than MTA, and 2- to 3-fold higher than n-HA/CS and HA, respectively. These increases were statistically significant compared with RM (P<0.001 for HA, P<0.001 for n-HA/CS, P<0.001 for CEM, P<0.001 for PC). Overall, ALP expression followed the order: CEM>PC (OM)>n-HA/CS>HA>MTA.

BSP expression: As shown in Fig. 4B, BSP expression increased significantly in all groups at both day 3 and day 6 compared with NC (P<0.001 for HA, P<0.001 for n-HA/CS, P<0.001 for CEM, P<0.001 for MTA, P<0.001 for PC). On day 6, all cement groups showed ~5-fold higher expression than RM, while OM reached ~10-fold. BSP expression was consistently highest in OM, with cement groups displaying comparable levels.

OC expression: Fig. 4C illustrates OC gene expression at days 3 and 6. On day 3, no group differed significantly from RM, indicating incomplete differentiation. By day 6, OC was significantly upregulated in all groups relative to NC (P=0.035 for HA, P<0.001 for n-HA/CS, P<0.001 for CEM, P=0.014 for MTA, P<0.001 for OM). Fold increases at day 6 were ~6-fold for HA, 12-fold for n-HA/CS, 10-fold for CEM, 6-fold for MTA, and 14-fold for PC. Overall, OC expression ranked: PC(OM)>n-HA/CS>CEM>HA≈MTA.

Discussion

This study evaluated the biological properties of two calcium silicate–based biomaterials (MTA and CEM) and two hydroxyapatite-based cements (HA and n-HA) on osteoblast-like Saos-2 cells, focusing on viability, attachment, proliferation, and differentiation. The rationale was to assess whether the diverse calcium constituents of CEM, the calcium ion release from MTA, the reduced particle size of n-HA, or the hydroxyapatite formation capacity of HA could differentially influence osteogenic responses.

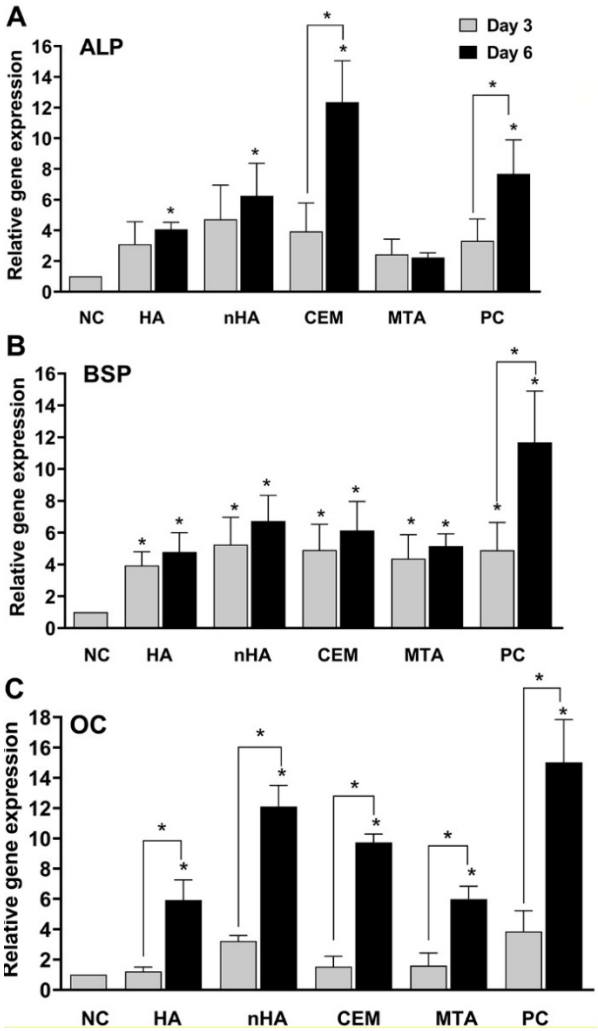

Figure 4. Relative gene expression of osteogenic markers in Saos-2 cells exposed to cement extracts at 3 and 6 days: *A)* alkaline phosphatase (ALP); *B)* bone sialoprotein (BSP); and *C)* osteocalcin (OC). Controls: NC (negative control, no differentiation) and PC (positive control, OM: osteogenic medium; induced differentiation). Data are presented as mean±SD from three independent experiments (n=3). P<0.05. Asterisks above columns indicate significance versus RM, while stars linked by horizontal lines indicate significance between experimental groups

The MTT assay demonstrated that none of the tested cements exhibited cytotoxicity, with all groups maintaining viability of greater than 70%, in accordance with ISO 10993-5 standards. Direct exposure experiments revealed that CEM significantly enhanced proliferation compared with other groups, consistent with previous reports showing that cell adhesion, proliferation, and differentiation are strongly modulated by both the physicochemical properties and ionic release of biomaterials [3, 20]. CEM contains a heterogeneous mixture of calcium compounds, including calcium silicate and calcium phosphate, which provide a reservoir of calcium and phosphorus ions that contribute to the precipitation of hydroxyapatite [21]. This may account for its superior support of proliferation compared with MTA, despite reports of similar overall biocompatibility [22-24]. The discrepancy with earlier findings may be attributable to differences in cell types used for evaluation.

Interestingly, HA outperformed n-HA/CS in short-term indirect assays, whereas n-HA/CS showed greater effects in long-term direct cultures. This pattern suggests that nano-scale surface topography enhances anchorage and differentiation once cells establish contact with the scaffold, consistent with previous evidence that n-HA supports improved osteoblast growth and survival compared with micro-HA [25]. Since ion release and hydroxyapatite formation are recognized determinants of bioactivity, these mechanisms likely underlie the favorable osteogenic responses observed in both CEM and n-HA/CS.

SEM images corroborated these findings, showing robust adhesion, ECM secretion, and cytoplasmic extensions across all groups, with particularly extensive coverage in the n-HA and CEM groups. The triangular surface particles and rougher topography of CEM may explain its enhanced cellular proliferation, as surface roughness is a well-established modulator of cell–material interactions [26]. The n-HA/CS supported strong anchorage and filopodia formation, consistent with its promotion of differentiation observed in qRT-PCR assays.

Gene expression analysis further supported these observations. CEM induced the highest upregulation of ALP, an early differentiation marker, whereas MTA consistently showed the lowest ALP expression, aligning with reports that MTA does not significantly enhance ALP activity in the first week of culture [27]. By contrast, both CEM and n-HA/CS strongly upregulated BSP and OC, mid- and late-stage markers of differentiation, indicating their potential to promote mineralized tissue formation. BSP is known to nucleate hydroxyapatite crystals and plays a central role in the early mineralization of bone and cementum [28], although its regulation remains incompletely understood [29]. The marked upregulation of BSP and OC observed here highlights the osteogenic potential of CEM and n-HA/CS compared with MTA. These results are consistent with previous studies demonstrating the importance of calcium silicate hydration products and bioactive ion release in activating signaling pathways for odontogenic and osteogenic differentiation [30, 31].

Osteocalcin is widely recognized as a late marker of osteoblastic differentiation and mineralization. It plays a critical role in regulating bone matrix maturation and mineral deposition. In this study, OC expression significantly increased by day 6 in all groups, confirming progression from proliferation to terminal differentiation. The highest levels were observed in

OM (positive control), followed by n-HA/CS and CEM, consistent with their ability to enhance osteogenic activity. Previous studies have reported that OC is upregulated when pre-osteoblasts fully differentiate, often alongside BSP, reflecting active mineralization [32]. Our findings align with these observations, as both OC and BSP were strongly induced in CEM- and n-HA/CS-treated cells, suggesting that these materials accelerate the transition of osteoblast-like cells toward a mature, mineralizing phenotype. In contrast, MTA induced a comparatively modest increase in OC, consistent with earlier reports showing delayed or limited stimulation of this marker [33]. Thus, OC expression in this model highlights the superior ability of CEM and n-HA to promote late-stage osteogenesis.

CEM cement demonstrated consistent and superior biological performance across assays. It supported the highest cell proliferation in direct contact experiments and induced robust ALP expression, indicating strong early osteogenic stimulation. The enhanced bioactivity of CEM may be attributed to its unique composition, which includes diverse calcium compounds (e.g., calcium silicate and calcium phosphate) that release biologically active ions such as Ca^{2+}, Si^{4+}, and PO_4^{3-} [21]. These ions are known to promote cell attachment, stimulate proliferation, and initiate hydroxyapatite nucleation, thereby enhancing cell–material interactions [34]. Moreover, SEM analysis revealed favorable surface roughness and particle morphology in CEM, which are recognized modulators of adhesion and extracellular matrix secretion. Previous studies have reported comparable overall biocompatibility and clinical effectiveness of CEM and MTA [3, 9, 20, 35, 36]; however, our data demonstrate that CEM provides superior proliferative and osteogenic responses, particularly in ALP and BSP upregulation. This suggests that the physicochemical properties of CEM confer advantages over MTA, positioning it as a promising candidate for clinical applications in regenerative endodontics.

While HA also supported osteogenic gene expression, its effects were generally weaker than the n-HA/Chitosan composite and CEM, underscoring how particle size and composite formulation substantially influence bioactivity. MTA, though long regarded as a "gold standard" biomaterial, consistently ranked lowest in our assays. It is important to note that this study utilized Angelus MTA, which possesses distinct bio-physicochemical properties from the original ProRoot MTA, which may contribute to the observed differential osteogenic response, possibly through variations in ion release kinetics or surface characteristics.

This study is limited by its *in vitro* design and use of Saos-2 cells, which, despite being a reliable osteoblast-like model, do not fully replicate the behavior of primary osteoblasts or dental pulp stem cells. Future studies should incorporate additional biomarkers, primary cell cultures, and *in vivo* models to confirm and extend these findings.

Conclusion

All tested cements (MTA, CEM, HA, n-HA/CS) exhibited acceptable biological properties on Saos-2 cells, showing no cytotoxicity, adequate adhesion, and induction of osteogenic differentiation. Among them, CEM promoted the highest cell proliferation, while both CEM and n-HA induced stronger osteogenic marker expression compared with MTA and HA. These findings suggest that CEM and n-HA/CS hold promise as bioactive candidates for hard tissue regeneration; however, further *in vivo* studies are required for clinical validation.

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Conflict of interest
None.

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Authors' contributions
Conceptualization: MT/SA/HN; Methodology: MT/HN/ZF/SM; Formal Analysis and Investigation: MT/SA; Writing-Original Draft Preparation: ME; Writing-Review and Editing: MT/SA/ME; Supervision: MT/AV/HN. All authors read and approved the final manuscript.

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