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Cytotoxicity and bioactive potential of new root repair materials for use with BMP-2 transfected human osteoblast cells

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Abstract: Modified formulations of calcium silicate repair materials with additives have been developed to enhance handling, consistency, biocompatibility and bioactivity. Considering the relevance of osteoblastic cell response to mineralized tissue repair, human osteoblastic cells (Saos-2 cells overexpressing BMP-2) were exposed to mineral trioxide aggregate (MTA) (with calcium tungstate - $CaWO_4$), MTA HP Repair, Bio-C Repair and Bio-C Pulpo. Cell viability was assessed by 3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide (MTT) and neutral red (NR), and cell death, by flow cytometry. Gene expression of bone morphogenetic protein 2 (BMP-2), runt-related transcription factor 2 (RUNX-2), and alkaline phosphatase (ALP) osteogenic markers were evaluated by real-time polymerase chain reaction (RT-qPCR). ALP activity and alizarin red staining (ARS) were used to detect mineralization nodule deposition. Bioactive cements presented no cytotoxic effect, and did not induce apoptosis at the higher dilution (1:12). MTA, Bio-C Repair and Bio-C Pulpo exhibited higher ALP activity than the control group ($P < 0.05$) after 7 days. MTA, MTA HP and Bio-C Pulpo affected the formation of mineralized nodules ($p < 0.05$). Exposure to all cement extracts for 1 day increased BMP-2 gene expression. RUNX-2 mRNA was greater in MTA, MTA HP and Bio-C Repair. MTA, MTA HP and Bio-C Pulpo increased the ALP mRNA expression, compared with BMP-2 unexposed cells ($p < 0.05$). Calcium silicate cements showed osteogenic potential and biocompatibility in Saos-2 cells transfected BMP-2, and increased the mRNA expression of BMP-2, RUNX-2, and ALP osteogenic markers in the BMP-2 transfected system, thereby promoting a cellular response to undertake the mineralized tissue repair.

Keywords: Endodontics; Biocompatible Materials; Cell Survival; Cell Proliferation; Bone Morphogenetic Protein 2

Introduction

An ideal root repair material should adhere and adapt to the dentinal walls, prevent leakage of microorganisms, and present biocompatibility and bioactive properties to induce tissue repair, or stimulate cell

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differentiation and regeneration.[1] Mineral trioxide aggregate (MTA) is the most common biomaterial, composed primarily of 53.1% tricalcium silicate, 22.5% dicalcium silicate, and bismuth oxide (Bi_2O_3) as a radiopacifier.[1] Radiopacifying agents have been studied to replace Bi_2O_3 in new tricalcium silicate-based cements.[2] Recently, Angelus Manufacturer (Angelus, Londrina, PR, Brazil) changed the MTA formulation and replaced Bi_2O_3 with calcium tungstate ($CaWO_4$). This substitution prevents tooth staining, and improves physicochemical properties.[3] MTA Repair HP (MTA "High Plasticity," Angelus, Londrina, PR, Brazil) is a bioceramic repair material with high plasticity, and has suitable physicochemical and biological properties.[4]

Premixed bioceramic materials are ready-to-use cements that promote biocompatibility and bioactivity.[5] Bio-C Repair (Angelus) is a calcium silicate repair material with zirconium oxide as a radiopacifier, proposed for direct pulp capping, pulpotomy or apexification, and has shown biocompatibility with periodontal ligament stem cells (hPDLSCs).[5] Bio-C Pulpo (Angelus) is a bioceramic repair cement, composed of calcium silicate powder and water, plasticizer and calcium chloride. Bio-C Pulpo has a fast setting time and is biocompatible.[6]

Bone tissue engineering has made substantial advances in the repair of massive bone defects, induced by both growth factors and seed cells differentiated into osteoblasts.[7] Bone morphogenetic protein-2 (BMP-2) has potent osteogenic ability, which can induce bone and cartilage formation both *in vivo* and *in* vitro.[8] Saos-2 cells overexpressing bone morphogenetic protein 2 (BMP-2) refers to a system previously developed by the authors to test the osteogenic activity of dental materials.[9] MTA, tri-calcium phosphate ceramics (TCP), and polyphasic calcium phosphates (Poly-CaP) are Ca^{2+} based materials with different compositions and levels of Ca^{2+} release.[10] Studies on these materials have shown that Ca^{2+} plays an essential role in mineralization by promoting cellular proliferation and differentiation.[10,11] In dental pulp stem cells (DPSCs), Ca^{2+} was found to increase BMP2 expression via calcium channels, and the extracellular signal-regulated kinases (Erk) pathway.[12]

The aim of this study was to investigate the osteogenic potential of certain bioceramic materials, their biocompatibility, bioactivity, mineralized nodule production, and mRNA expression of osteogenic markers, namely BMP-2, runt-related transcription factor 2 (RUNX-2), and alkaline phosphatase (ALP); and their cell differentiation induced by MTA Angelus, MTA HP, Bio-C Repair and Bio-C Pulpo, by using Saos-2 cells overexpressing BMP-2.

Methodology

Cell culture

Saos-2 osteoblastic-transfected cells (Saos-BMP-2) and Saos-pcDNA 3.1 (+) empty vector cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (non-osteogenic medium - Sigma-Aldrich, St. Louis, USA), supplemented with 10% fetal bovine serum (FBS), (Gibco/Invitrogen, Waltham, USA), penicillin (Gibco/Invitrogen) (100 IU/mL), and streptomycin (Gibco/Invitrogen) (100 µg/mL), under 5% CO_2, in a 95% humidified atmosphere at 37^{0}C until confluency. Next, the cells were plated and allowed to attach for 24 hours. The culture medium was replaced, and the cells were then incubated with cement extracts at 1:1, 1:6, 1:12 and 1:24 dilutions to test viability [1:12 was used for activity experiments]; DMEM non-osteogenic medium was used as a control. The cell culture medium was supplemented with 50 µg/mL L-ascorbic acid (Sigma) and 5mMβ-glycerophosphate (Sigma), and used to perform the osteoinductive assay (osteogenic medium).

Transfection

A gene sequence encoding BMP-2 was synthesized (Epoch Life Science, Missouri, USA) and cloned into the eukaryotic expression vector pcDNA3.1 (+) (Invitrogen, Carlsbad, USA). The transfection of Saos-2 cells was performed using the liposome method and Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instructions. Cell clones were isolated and expanded for characterization by polymerase chain reaction (PCR) and Western blotting analysis.[9]

Table. Materials and proportions used.

Material	Manufacturer	Proportion
MTA	Mineral Trioxide Aggregate. Tricalcium silicate, dicalcium silicate and calcium tungstate. Liquid: distilled water (Angelus, Brazil)	1g/ 300μL
MTA HP	Tricalcium silicate, dicalcium silicate, tricalcium aluminate, calcium oxide and calcium tungstate. Liquid: Water and plasticizer (Angelus, , Brazil)	1g/ 300μL
Bio-C Repair	Calcium silicate, calcium oxide, zirconium oxide, iron oxide and silicon dioxide. Liquid: dispersing agent (Angelus,, Brazil)	Ready for use
Bio-C Pulpo	Calcium silicate, calcium aluminate, calcium hydroxide, zirconium oxide, calcium fluoride, silicon dioxide and iron oxide. Liquid: distilled water, calcium chloride, methylparaben and plasticizer (Angelus, Brazil)	1 dose/ 3 drops

Preparation of the cement extracts

The materials evaluated in this study, their proportions and manufacturers are described in Table. After manipulation, the materials were weighed (0.5 g), placed in empty wells of 12-well culture plates and incubated for 15 h at 95% humidity, 5% CO2, and 37°C to ensure their polymerization. They were then exposed to ultraviolet light (UV) under laminar flow for 30 min for disinfection Afterwards, 5 mL of DMEM serum-free medium was added to each well, followed by reincubation for 24 h to obtain the experimental extracts. All the extracts were further diluted serially at 1:1, 1:6, 1:12 and 1:24 (v:v) in DMEM serum-free medium to observe the dose–response relationship.

Cell viability assays

The cell viability rate was determined by 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-tetrazolium bromide (MTT). This assay was performed by plating $1x10^4$ cells/well in a 96-well plate (Corning, Union City, USA). The cells were exposed to cement extracts or DMEM non-osteogenic serum-free medium (negative control) and 1 mM hydrogen peroxide (positive control). After 24 hours, the culture medium was changed to DMEM containing 0.55 mg/mL MTT solution, and the plates were incubated for an additional 4 hours at 95% humidity, 5% CO2, and 37°C. Each well was washed once with 100 μL of phosphate-buffered saline (PBS), after which 100 μL acid-isopropyl alcohol (isopropyl alcohol, 0.04 N) was added to solubilize the formazan. The optical density (OD = 570 nm) was measured using an automated microplate reader. Three independent experiments were performed in triplicate for each experimental group. For the neutral red (NR) cytotoxicity assay, the extracts were replaced with 0.1 mL DMEM containing 50 μg NR/mL (Sigma-Aldrich), followed by incubation at 37°C, 95% humidity and 5% CO_2 for 3 h. Then, the content of the well was removed, and the colorimetric product was solubilized in 100 μL of an ethanol solution mixture (50% ethanol and 1% acetic acid) (Sigma-Aldrich). The optical densities of the solutions were measured in a spectrophotometer (Elx800) at 570 nm. Again, three independent experiments were performed in triplicate for each experimental group, to establish the dose-response for further bioactivity analyses.

Detection of apoptosis

The effect of the materials on Saos-BMP-2 transfected cell apoptosis was assessed by determining the percentage distribution of living cells (LC) (annexin-/PI-), initial apoptosis (IA) (annexin+/PI-), late apoptosis or necrotic (N) cell populations (annexin+/PI+, and annexin-/PI+), exposure of phosphatidylserine on cell membrane (early stage), and nuclear membrane permeability (late-stage or necrosis). The last assay determined flow cytometry using an Annexin V detection kit, according to the manufacturer's instructions (Annexin V-PE Apoptosis Detection Kit I, BD Biosciences, Pharmingen, San Jose, USA). Cells (1×10^6 cells/well) were plated in a 24-well plate (Corning), and exposed to the cement extracts (1:12), or DMEM non-osteogenic serum-free medium (negative control group), and 1 mM hydrogen peroxide (positive control) for 24 hours. Saos-BMP-2 transfected cells treated with H_2O_2 (1 mM) were used as the positive control, and the cells exposed to the non-osteogenic serum-free medium

were the negative control. Three independent experiments were performed in sextuplicate for each experimental group and outcome. A total of 100,000 events were analyzed for each sample of this experiment.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was evaluated by using a commercial kit (Labtest; Lagoa Santa, Brazil). A total of $1x10^4$ cells/well were plated in 96-well culture plates and exposed to the cement extracts (1:12), and/or to DMEM non-osteogenic serum-free medium (control group) for one, three and seven days. Absorbance was measured at 590 nm in a spectrophotometer. Three independent experiments were performed in sextuplicate for each experimental group and outcome. The data were expressed as ALP activity normalized by the total protein in the respective time interval of the culture.

Alizarin red staining (ARS)

Saos-BMP-2 transfected cells ($0,2x10^4$ cells/well) were plated in 24-well culture plates (4 wells/solution). After 21 days of cell culture in osteogenic medium with and without the cements extracts (1:12), the cells were washed with PBS, fixed with 10% paraformaldehyde (Sigma), and then stained with 2% alizarin red S (pH 4.1). Next, mineralization was quantified by dissolving alizarin red with 500 µL of 10 % cetylpyridinium chloride (Sigma-Aldrich) solution per well for 15 min, shaking gently at room temperature. Four aliquots of 100 µL of the stained solutions of each well were transferred to wells of 96-well plates, and read at 562 nm in a microplate reader (ELx800; Bio-Tek Instruments, Winooski, USA). Three independent experiments were performed in quadruplicate for each experimental group.

Quantitative real time PCR (RT-qPCR)

Saos-BMP-2 transfected cells ($6x10^4$ cells/well) were plated in 6-well plates (Corning). After 24 hours of exposure to the cement extracts (1:12) and/or to DMEM non-osteogenic serum-free medium (control group), the RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, USA), according to the instructions provided by the supplier. The gene expression was analyzed by RT-qPCR (StepOne, Applied Biosystems, Foster City, USA), using TaqMan chemistry with pre-designed primers and probe sets (Gene Expression Assays, Applied Biosystems). The primer/probe references are: BMP- 2 (Hs00154192_m1); RUNX-2 (Hs01047973_m1); ALP (Hs01029144_m1) and GAPDH (Hs02758991_g1) (Applied Biosystems, Life technologies). Triplicates were prepared for each reaction, and the experiment was repeated three times independently. The levels of target gene expression for each sample group were calculated using the $\Delta\Delta$Ct method, and compared with the control.

Statistical analysis

The results were analyzed using GraphPad Prism (GraphPad Software, San Diego, USA), with a 5% significance level. The statistical tests applied were one-way analysis of variance (ANOVA) and Turkey's post-test, two-way ANOVA and Bonferroni's post-test.

Results

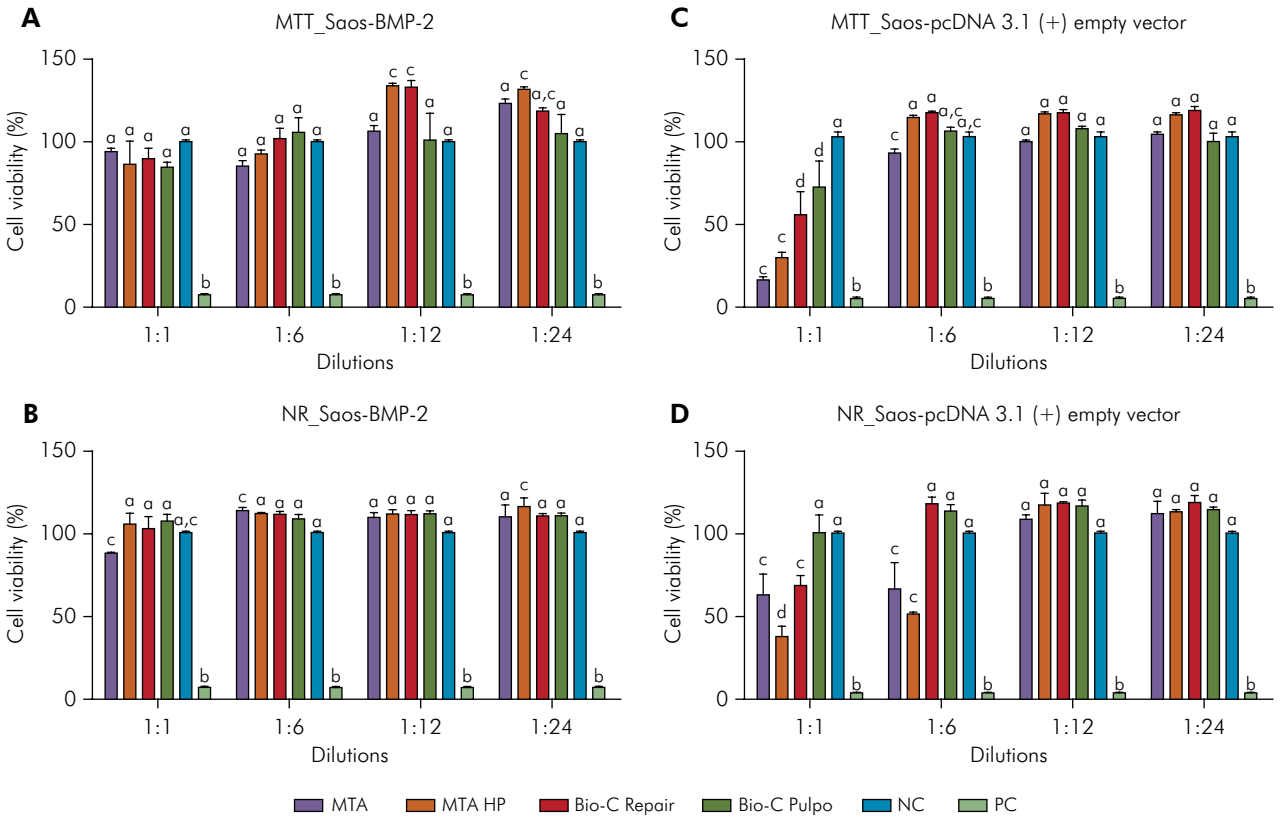
Cell viability

The Saos-BMP-2 transfected cells presented no cytotoxic effects when evaluated by MTT (Figure 1a) and NR assays, and MTA assay showed low cell viability ($p < 0.05$) in 1:1 dilution (NR), in relation to the negative control (Figure 1b). MTA HP induced high cell ($p < 0.05$) viability in 1:12 and 1:24 dilutions (MTT), compared with the negative control (Figure 1a).

In the MTT assay, Saos-pcDNA 3.1 (+) empty vector cells exposed to all cements exhibited low cell viability in 1:1 dilution, compared with the negative control Figure 1c). MTA and MTA HP showed low cell viability ($p < 0.05$) in 1:1 and 1:6 dilutions (NR), in relation to the negative control (Figure 1d). In the 1:12 and 1:24 dilutions, the cells were similar to the unexposed control ($p > 0.05$) in both assays (Figures 1c and 1d).

Detection of apoptosis

Exposure of Saos-BMP-2 transfected cells to the cement extracts for 24 hours did not induce apoptosis (Figure 2a). The live cell population group was higher than (MTA, MTA HP and Bio-C Pulpo) or similar to



NC: negative control group; PC: positive control.

Figure 1. Cell viability rate (%) according to MTT and neutral red (NR) assays in Saos-2 osteoblastic-transfected cells: (a and b) Saos-BMP-2 and (c and d) Saos-pcDNA 3.1 (+) empty vector, exposed to MTA, MTA HP, Bio-C Repair, Bio-C Pulpo and serum-free culture medium used as a control in different dilutions (1:1, 1:6, 1:12 and 1:24) for 24 h. Three independent experiments were performed in sextuplicate for each experimental group and outcome (n = 3/group). Bars with different letters represent a significant difference among the groups in each dilution. ANOVA, Bonferroni ($p < 0.05$).

(Bio-C Repair) the negative control. However, in Saos-pcDNA 3.1 (+) empty vector cells, there was low cell viability when exposed to Bio-C Repair and Bio-C Pulpo, compared with the negative control ($p < 0.05$). MTA HP, Bio-C Repair and Bio-C Pulpo ($p < 0.05$) induced apoptosis, compared with the negative group (Figure 2b). There was no increase in the necrotic cells in either of the transfected lines, compared with the positive control ($p < 0.05$).

ALP activity

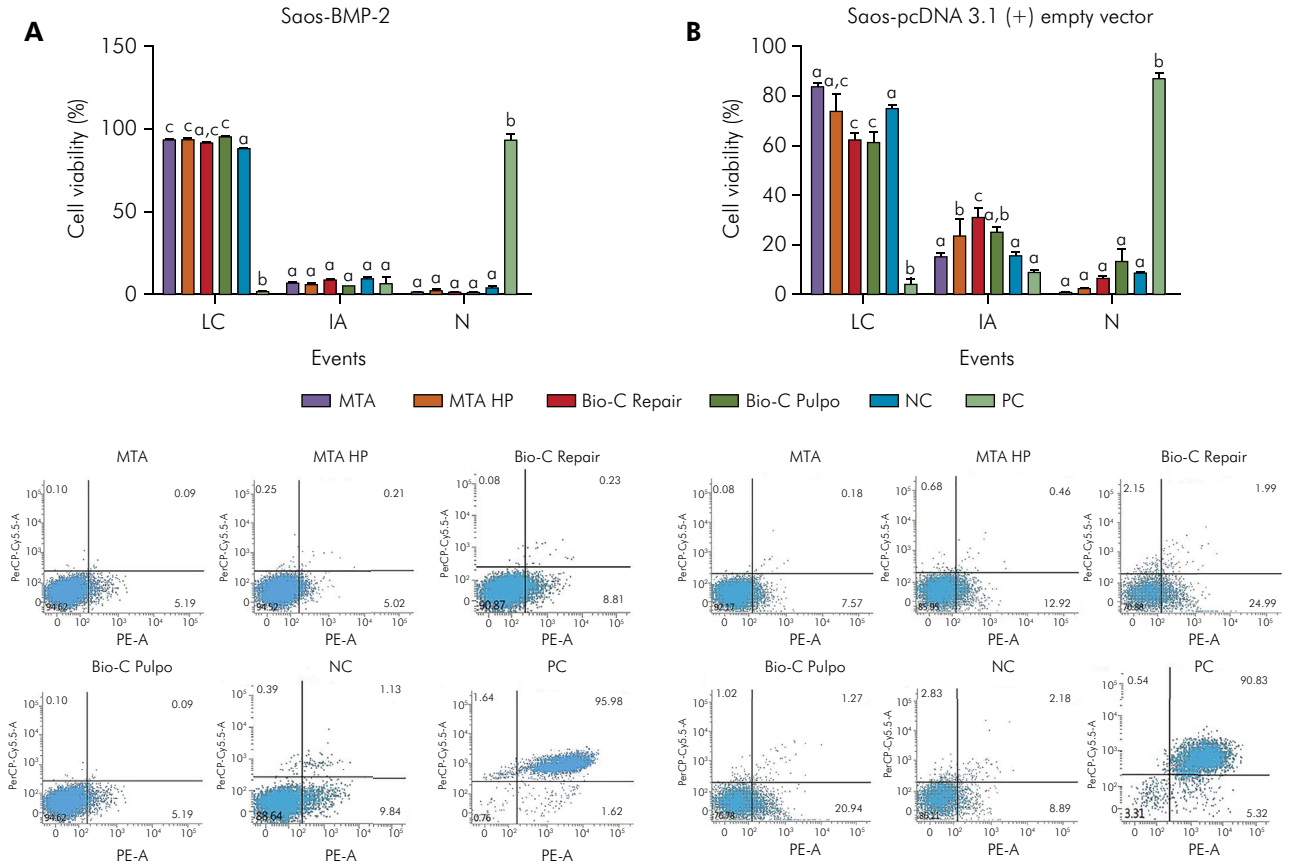
There were no cytotoxic effects in either of the transfected cell lines, in relation to the negative control, in all the periods evaluated (1–7 days). MTA and MTA HP had increased cell viability ($p < 0.05$) at 3 and 7 days, compared with the control (Figures 3a and 3c).

The Saos-BMP-2 transfected cells exposed to MTA, Bio-C Repair and Bio-C Pulpo exhibited the highest ALP activity, compared with the control group ($p < 0.05$) after 7 days of culture (Figure 3b).

MTA increased the ALP activity in Saos-pcDNA 3.1 (+) empty vector cells ($p < 0.05$) in all the periods evaluated, in relation to the control. Bio-C Pulpo induced an increase in ALP activity after 3 days, and Bio-C Repair had the lowest ALP activity ($p < 0.05$) in all the periods, compared with the control (Figure 3d).

Mineralization nodules

After 21 days of cell exposure, MTA, MTA HP and Bio-C Pulpo had a significantly ($p < 0.05$) greater stimulatory effect on the formation of mineralized nodules in Saos-BMP-2 transfected cells than the control group (Figure 4a). The same stimulatory effect



NC: negative control; PC: positive control.

Figure 2. Flow cytometry assay. (a) Viability and mortality by apoptosis or necrosis of Saos-2 osteoblastic-transfected cells (Saos-BMP-2) and (b) Saos-pcDNA 3.1 (+) empty vector after exposure to cement extracts at 1:12 dilution for 24 hours. Cells exposed to serum-free culture medium were the negative control, while cells treated with hydrogen peroxide (1 mM) were the positive control group. (b) Representative 2-dimensional flow cytometry dot plot of data derived from PE-AnV and 7 AAD stained Saos-2 osteoblastic-transfected cells of the control and the cement extract groups. Bars with different letters represent significant differences among groups in each population analyzed. The population of living cells (LC) (annexin-/ PI-), initial apoptosis (IA) (annexin+/ PI-), late apoptosis and/or necrotic (N) cells (annexin+/ PI+, and annexin-/ PI+) are represented at the bottom left, bottom right, top right and top left quadrants, respectively.

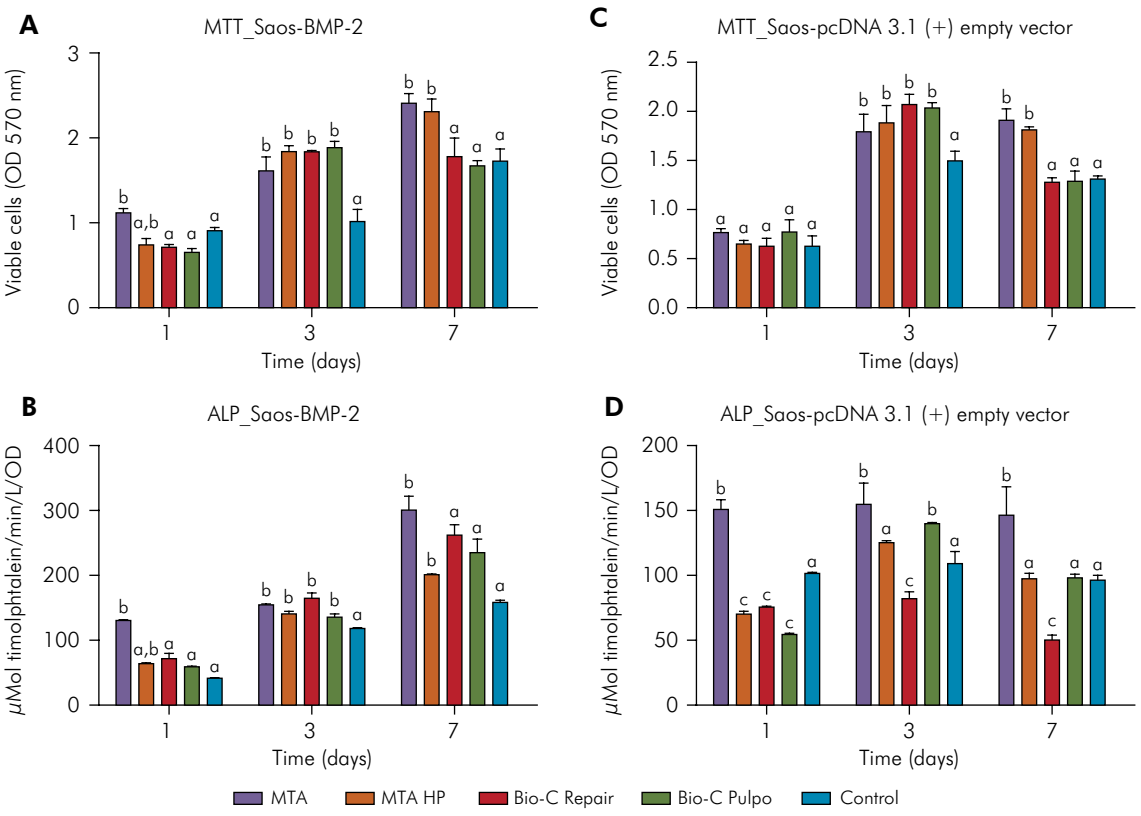
was observed in Bio-C Repair and Bio-C Pulpo in Saos-pcDNA 3.1 (+) empty vector cells ($P < 0.05$), in comparison with the control (Figure 4b).

RT-qPCR analysis

BMP-2 transfected cells exposed to all cement extracts for 1 day increased BMP-2 gene expression (~1000.0-fold increase; $P < 0.05$), but a decrease in BMP-2 mRNA expression was observed at 7 days, compared with unexposed BMP-2 transfected cells (Figure 5a).

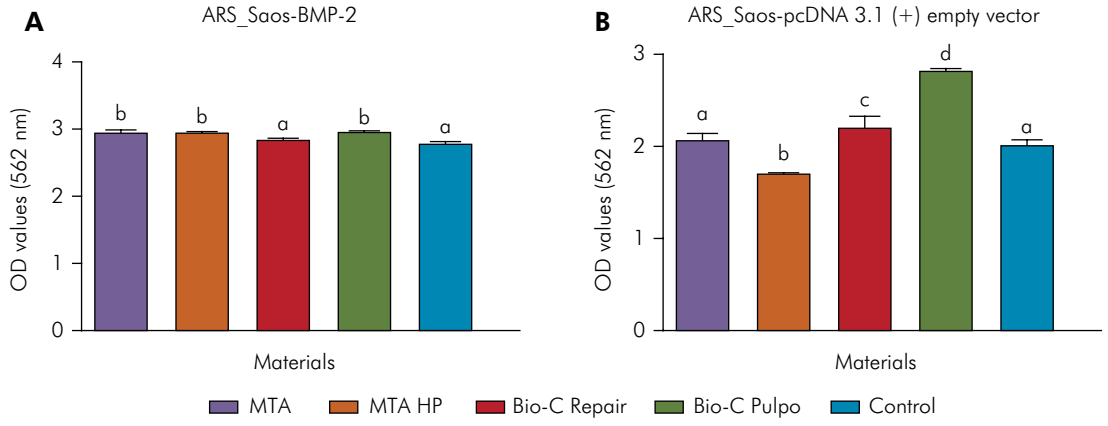
RUNX-2 mRNA was increased on the first day of exposure to MTA, MTA HP and Bio-C Repair (Figure 5a), particularly MTA, which was able to increase RUNX-2 mRNA in BMP-2 transfected cells (~3.0-fold increase; $p < 0.05$), in comparison to the unexposed cell group. There was no detected BMP-2 or RUNX-2 mRNA expression in Saos-pcDNA 3.1 (+) empty vector cells (Figure 5b).

ALP mRNA expression was increased in both the system transfected with BMP-2 and the Saos-pcDNA 3.1 (+) empty vector cells. ALP transcript increased ($p < 0.05$) only on the first day of MTA, MTA HP and Bio-C Pulpo exposure to BMP-2-transfected cells (~2.0-fold increase; $p < 0.05$), in relation to the unexposed BMP-2 transfected cells (Figure 5a). MTA and MTA



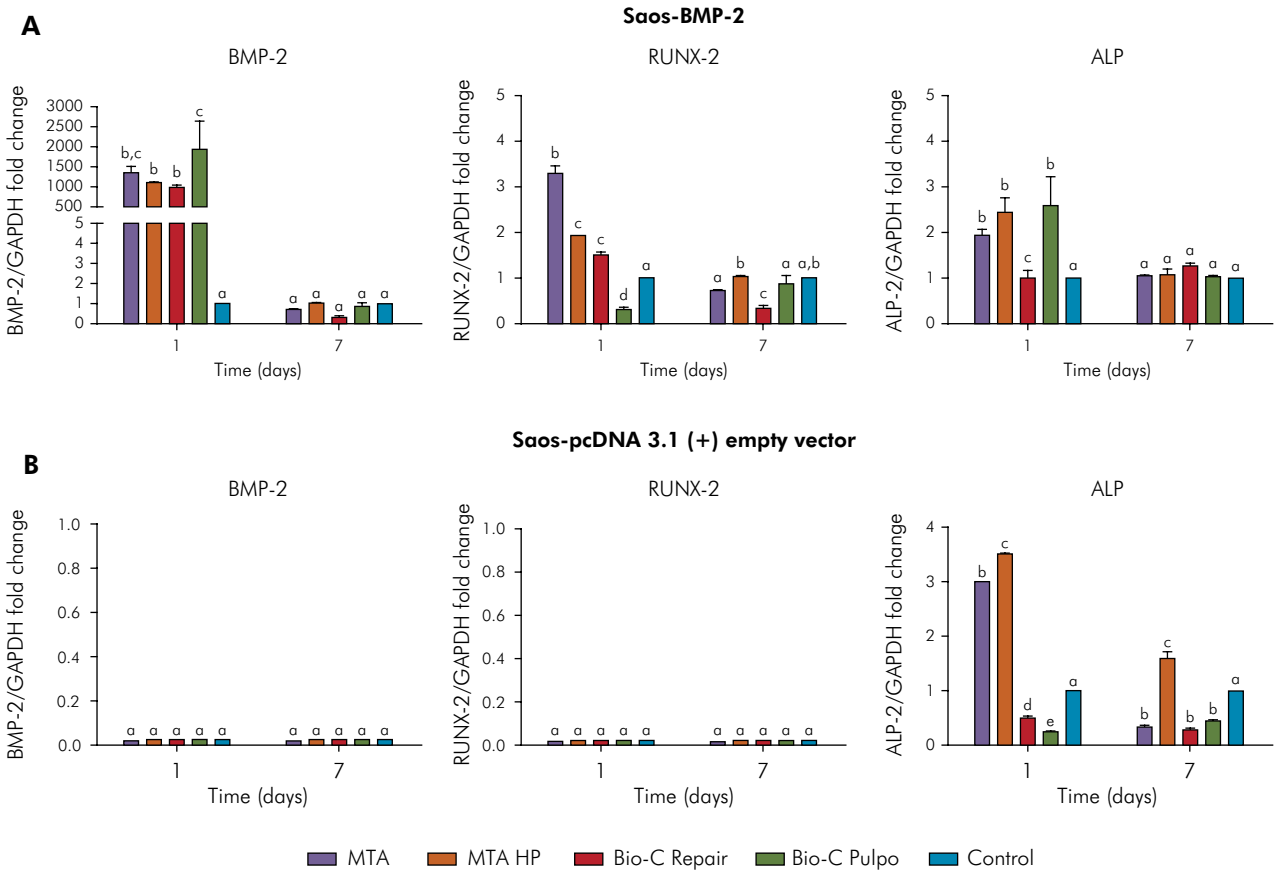
Bars with different letters represent significant differences among the groups. ANOVA, Bonferroni Control = cells exposed to serum-free culture medium.

Figure 3. Cell viability and ALP evaluation. (a and b) Saos-BMP-2 transfected cells and (c and d) Saos-pcDNA 3.1 (+) empty vector after exposure to MTA, MTA HP, Bio-C Repair, Bio-C Pulpo at 1:12 dilution, and to culture medium (control) for 1, 3 and 7 days, respectively. Three independent experiments were performed in sextuplicate for each experimental group and outcome (n = 3/group).



ANOVA, Tukey's test ($p < 0.05$). Control = cells exposed to osteogenic medium.

Figure 4. ARS statistical analysis after 21 days of exposure of the materials (MTA, MTA HP, Bio-C Repair, Bio-C Pulpo at 1:12 dilution) to (a) Saos-BMP-2 transfected cells and (b) Saos-pcDNA 3.1 (+) empty vector. Unexposed cells were the control group. Identical triplicates were prepared for each reaction, and the experiment was repeated three times independently (n = 3/group). Bars with different letters represent significant differences among the groups.



ANOVA, Bonferroni ($p < 0.05$). BMP-2: bone morphogenetic protein-2; RUNX-2: runt-related transcription factor 2; ALP: alkaline phosphatase genes. Control = cells exposed to culture medium.

Figure 5. mRNA expression levels of target genes in (a) Saos-BMP-2 transfected cells and (b) Saos-pcDNA 3.1 (+) empty vector exposed to MTA, MTA HP, Bio-C Repair, Bio-C Pulpo at 1:12 dilution and to culture medium (control) for 1 and 7 days. Identical triplicates were prepared for each reaction, and the experiment was repeated three times independently (n = 3/group). Bars with different letters represent significant differences among the groups.

HP induced a greater ALP mRNA expression (~3.0 fold increase; $p < 0.05$) on the first day of exposure to Saos-pcDNA 3.1 (+) empty vector cells, and MTA HP, on the seventh day of exposure, compared with the unexposed cells (Figure 5b).

Discussion

The aim of this study was to investigate the cytotoxic effects and osteoblast differentiation in BMP-2-transfected cells exposed to bioceramic root repair materials. Although MTA has often been used as the reference material,[13] there are no biological studies that have investigated its new formulation replacing Bi_2O_3 with $CaWO_4$, regarding biocompatibility and bioactive properties for inducing tissue repair.

The MTT assay measures succinate dehydrogenase mitochondrial enzyme activity, in which viable cells metabolically reduce the soluble yellow of tetrazolium salt to insoluble blue-violet formazan crystals. The absorbance of these crystals is proportional to the number of viable cells.[14] NR is a viability assay that determines how well viable cells can incorporate this dye into their lysosomes, where it accumulates when the cell membrane is intact.[15] More than one cell viability assay is required to double-check the possible cytotoxic effects of the materials. Moreover, indications of the toxicity mechanisms, and significant results can be identified by the simultaneous evaluation of different cell parameters.[16] The MTT and NR results showed that the cement extracts were not cytotoxic for Saos-BMP-2 transfected cells at the proposed

dilutions (1:1, 1:6, 1:12 and 1:24), and MTA assay, at a 1:1 dilution, as evaluated by NR. As in other studies,[17,18] our previous study showed that MTA was biocompatible with these BMP-2 transfected cells[9]. This indicates that the MTA used as a radiopacifier in this new formulation with $CaWO_4$ retained the same biocompatibility. MTA HP showed no cytotoxic effects, and had a better proliferation response at 72 hours than White MTA-Angelus (MTA-Ang), which contained Bi_2O_3 as a radiopacifier.[19] The authors reported that these findings may be attributed to the absence of Bi_2O_3 in the MTA HP composition. This is based on previous studies, which showed that Bi_2O_3 could have a cytotoxic effect on human osteoblast-like cells associated to dicalcium silicate cement, compared with the association without this radiopacifier.[20] The new Bio-C Repair and Bio-C Pulpo bioceramic cements also showed biocompatibility with Saos-BMP-2 transfected cells. Furthermore, Bio-C Repair was also biocompatible with these cells when it was associated to hPDLSCs.[5]

Some materials, such as MTA, do not induce significant apoptosis and/or necrosis in cell lineages.[13,21] In addition to showing absence of apoptosis, our results also showed increased cell proliferation for MTA, MTA HP and Bio-C Pulpo cements in cells that received an extra copy of the BMP-2 gene. Ferreira et al.[4] reported that MTA HP showed a low percentage of apoptotic or necrotic cells (< 3%). This increase in cell proliferation and absence of apoptosis observed with exposure of Bio-C Pulpo to the cell lineages may be related to the zirconium oxide (ZrO_2) incorporation as a radiopacifier. ZrO_2 has been shown to be biocompatible with low cytotoxicity.[22] A study showed that the high concentration of ZrO_2 material (10 mg/mL) in all its groups caused increased viability in NIH-3T3 fibroblasts, particularly after 12 and 24 h.[23] Lima et al.[24] reported that the replacement of Bi_2O_3 with ZrO_2 in Bio-C Pulpo did not change the clinical repair performance of the material regarding the pulpotomy of primary teeth, nor the tooth color. The setting time, compressive strength and radiopacity of the tested materials are in compliance with the standards for water-based dental cements[24,25]. Bio-C Repair also did not induce apoptosis, corroborating the findings of the López-García et al.[5] (2019) study.

ALP is expressed during the early maturation of osteoblasts, and plays an important role in the mineralization process.[26] In the present study, ALP activity on the first day was increased in the MTA group, and was stimulated by MTA, MTA HP and Bio-C Repair on the third day of exposure. No cytotoxicity was observed in relation to the untreated Saos-BMP-2 transfected cells. Higher ALP activity was observed in the MTA, Bio-C Repair and Bio-C Pulpo groups after 7 days of culture. MTA has been related to inducing high levels of ALP activity.[9,13,27] Bio-C repair has been reported as being able to induce biomineralization *in* vitro.[28]

The high pH of calcium silicate-based materials may be associated with the formation of calcium hydroxide and calcium silicate hydrogel.[29] Alkaline pH increases osteoblast ALP activity and antimicrobial activity.[30] Similar pH was observed for Bio-C Sealer and Bio-C Repair, when immersed in DMEM, with Bio-C Sealer releasing significantly more calcium and silicon ions than Bio-C Repair.[5] Bio-C Pulpo, Biodentine and TotalFill demonstrated calcium release, antibacterial activity, and an effect on cellular metabolic activity.[31]

The calcium deposition observed in Saos-2-BMP-2-transfected cells was consistent with the increase in ALP activity, especially in MTA, MTA HP and Bio-C Pulpo. Deposits of calcium have been reported when cells are exposed to MTA.[9,10,13,27,32]

Differences among the materials in Saos-2-BMP-2-transfected cell responses were observed at the gene level. BMP-2 is a cytokine, member of the transforming growth factor beta (TGF-β) superfamily, which plays a decisive role in bone formation, regeneration, and repair.[33] BMP2 activates BMP receptors and induces receptor-specific Smads (R-Smads), which can lead to a process of osteoblast differentiation through the up-regulation of transcription factors such as RUNX-2, Osterix (Osx), distal-less homeobox 5 (Dlx5), and Msh homeobox 2 (Msx2).[34] On the first day of Saos-BMP-2-transfected cell exposure, all the materials overexpressed the BMP-2 gene, particularly Bio-C Pulpo, owing to its ability to induce the highest BMP-2 mRNA expression, compared with the other materials. MTA is already known to be associated with an increase in BMP-2 transcripts,[11,13] but a significant

overexpression of BMP-2 has recently been detected in Saos-BMP-2-transfected cells.[9]

RUNX-2 is the first osteogenic transcription marker expressed by the osteoblast cell line, and coordinates multiple signaling pathways related to the differentiation of osteoblasts[35]. BMPs interact with RUNX-2, and these interactions are important to achieve osteoblast differentiation and bone formation.[34] MTA, MTA HP and Bio-C Repair materials stimulated an increase in mRNA expression of RUNX-2 during one day of exposure to Saos-BMP-2-transfected cells. The RUNX-2 marker was up-regulated when the stem cells of the apical papilla (SCAPs) were exposed to MTA for 7 days.[32] There was no BMP-2 or RUNX-2 mRNA expression in cells transfected with the empty vector. Osteogenic induction by BMPs has been studied extensively. Most of these, particularly BMP-2, are expressed in very low concentrations by mammalian osteoblastic cell types *in* vitro.[36] Saos-BMP-2 transfected cells possess an extra BMP-2 gene copy that can simulate the mechanism by which only one cell phenotype is activated by BMP-2. BMP-2 intracellular receptor Smads, particularly Smad5, activate osteoblast-essential genes, including the transcription factors required for *in vivo* bone formation by RUNX-2 (Cbfa1/ AML3) and Osterix.[37]

ALP is one of the major enzymes expressed during the early maturation of osteoblasts, and plays an important role in mineral deposition.[26] All the tested materials induced an increase in ALP mRNA, especially MTA, MTA HP and Bio-C Pulpo, on the first day of exposure to Saos-BMP-2-transfected cells. In Saos-pcDNA 3.1 (+) empty vector cells, MTA and MTA HP also induced an increase in ALP mRNA expression. Our previous study[9] showed that MTA increased the ALP transcript when Saos-BMP-2-transfected cells were exposed to MTA prepared with osteogenic medium. In this new version, MTA induced an ALP mRNA overexpression with no possible cumulative effect of osteogenic factors from the medium. Rat mesenchymal stem cells (MSCs) treated with ProRoot MTA exhibited upregulation of the ALP gene expression on the first day of exposure.[38] In agreement with our results, another study reported that ALP mRNA expression was greater in fluoride-treated Saos-2 cells via the BMP/Smad signaling pathway.[39]

Bio-C Pulpo composition is similar to other repair materials (MTA, MTA-HP and, Bio C-Repair), but has some added components, such as zirconia oxide radiopacifier; silicon oxide to control viscosity; iron oxide as a pigmenting agent; and liquid plasticizer and calcium chloride to promote adequate physicochemical properties and accelerate the setting time (Angelus). An immunohistochemical study reported that Bio-C Pulpo induced immunolabelling of osteogenic markers, such as osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP), similar to White MTA-Ang.[9] The traditional use of $Ca(OH)_2$ to achieve apexification is being gradually replaced by the use of MTA and new bioceramic materials.[40,41] As such, Bio-C Pulpo also showed potential for use in endodontic therapy with clinical indications other than those recommended by the manufacturer, thus corroborating our findings related to its ability to stimulate mineralization. It may represent an effective alternative for apexification procedures and tissue regeneration. This study should be considered an initial assessment of the osteogenic effect of Bio-C Pulpo. Different methods, including experimental animal investigation and clinical studies, are suggested to further the research into Bio-C Pulpo.

Conclusion

This study revealed that bioactive cements were biocompatible with Saos-BMP-2 transfected cells, induced osteogenic bioactivity, and enhanced mRNA expression of BMP-2, RUNX-2 and ALP osteogenic markers in a BMP-2 transfected system. The replacement of Bi_2O_3 with $CaWO_4$ in MTA showed no resulting alterations, and represented an improvement in the biological properties of MTA. Bio-C Pulpo stimulated mineralization, and is an effective potential alternative for use as a reparative material.

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