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Oxidative stress induced by self-adhesive resin cements affects gene expression, cellular proliferation and mineralization potential of the MDPC-23 odontoblast-like cells

Marcela Maciel Palacio Alvarez[a], *Rafael Guzella de Carvalho*[a], *Silvana Coelho de Arruda Barbosa*[b], *Mackeler Ramos Polassi*[b], *Fábio Dupart Nascimento*[c], *Paulo Henrique Perlatti D'Alpino*[b,*], *Ivarne Luis dos Santos Tersariol*[a,**]

[a] *Department of Biochemistry, Federal University of São Paulo, São Paulo, Brazil*
[b] *Biotechnology and Innovation in Health Program, Universidade Anhanguera de São Paulo (UNIAN-SP), São Paulo, SP, Brazil*
[c] *Interdisciplinary Center of Biochemistry Investigation, University of Mogi das Cruzes, Mogi das Cruzes, SP, Brazil*

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ABSTRACT

Objective. Clinical issues have been raised about problems related to cytotoxic effects caused when applying self-adhesive cement. It was hypothesized that byproducts eluted from self-adhesive cements modulate oxidative stress response, the gene expression of signaling pathways of inflammatory process/transcriptional activators, and the expression and activity of interstitial collagenases, and modify the phenotypic characteristics of cellular proliferation and mineral deposition in odontoblastic-like cells.

Methods. Cements (MaxCem Elite [MAX] and RelyX U200 [U200])) were mixed, dispensed into moulds, and photoactivated according to the manufacturers' instructions. Immortalized rat odontoblast-like cells (MDPC-23) were cultured and exposed to polymerized specimens of cements for 4 h. Reactive oxidative specimen production and quantification of gene expression were evaluated. Cell proliferation assay and alizarin red staining were also performed to evaluate the disturbance induced by the cements on cellular proliferation and mineralization.

Results. Despite their cytotoxic effects, both self-adhesive cements influenced the metabolism in the odontoblast cells on different scales. MAX induced significantly higher oxidative stress in odontoblast cells than U200. Gene expression varied as a function of exposure to self-adhesive cements; MAX induced the expression of pro-inflammatory cytokines such as TNF-α, whereas U200 downregulated, virtually depleted TNF-α expression, also inducing overexpression of the transcriptional factor Runx2. Overexpression of heme oxygenase-1 (HO-1) and thioredoxin reductase 1 (TRXR1) occurred after exposure to both

* *Corresponding author at*: Universidade Anhanguera de São Paulo — UNIAN SP, Programa de Pós-Graduação em Biotecnologia e Inovação em Saúde, Av. Raimundo Pereira de Magalhães, 3305, São Paulo, SP, 05145-200, Brazil.
** *Corresponding author at*: Department of Biochemistry, Molecular Biology Division, Federal University of São Paulo (UNIFESP), Rua três de maio, 100-4th floor, São Paulo, SP, 04044-020, Brazil.
E-mail addresses: mpalvarez.marcela@gmail.com (M.M.P. Alvarez), rafaelguc@hotmail.com (R.G. Carvalho), silvanacoelhobio@gmail.com (S.C.A. Barbosa), mackeler@gmail.com (M.R. Polassi), fdnascimento@gmail.com (F.D. Nascimento), paulodalpino@gmail.com (P.H.P. D'Alpino), ivarne.tersariol@gmail.com (I.L.S. Tersariol).
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cements, antioxidant genes that are downstream of Keap1-Nrf2-ARE system. MAX significantly induced the overexpression of collagenase MMP-1, and U200 induced the expression of gelatinase MMP-2. MAX significantly inhibited cell proliferation whereas U200 significantly activated cell proliferation. Alizarin red staining revealed significantly decreased mineral deposition especially when exposed to MAX.

Significance. These results support the hypothesis that byproducts of different self-adhesive cements play important roles in the highly orchestrated process which ultimately affect the cellular proliferation and the mineral deposition in odontoblastic-like cells, possibly delaying the reparative dentin formation after cementation of indirect restorations, especially on recently exposed dentin preparations.

1. Introduction

Self-adhesive cements contain self-etching functionalized monomers that significantly reduce the pH and demineralize the tooth structure, promoting micromechanical adhesion [1,2]. These acid-functionalized monomers (i.e. Phenyl-P, 4-META, 10-MDP) form secondary reactions (ionic and covalent interactions) between the cement and the tooth structure, converting these substances into a salt complex formed by calcium and the acid-modified monomers, thereby establishing chemical bonds with the tooth structure [1]. The key point is to simplify the luting procedures of indirect restorations while maintaining the good performance of conventional cements [3].

Concerns have been raised about the chemical composition of restorative materials and their application protocols to the dentinal tissue, especially regarding the particularity of self-adhesive cements [4–7]. After etching the dental tissues, a dynamic process occurs in which the acidity of these acid-functionalized monomers is neutralized concurrently with the polymerization process, developed during and after the light exposure [8]. By impacting the polymerization process, (co)monomers and other byproducts can be eluted from polymerized dental methacrylate-based materials, potentially affecting both the biocompatibility and the structural stability of the restoration [8,9].

The biological responses of the pulpal tissues after restorative procedures include cytotoxic effects, such as induced oxidative stress–mediated pulp cell death, inflammatory mediator overexpression, and depletion of glutathione peroxidase and superoxide dismutase enzymes [10]. Eluted byproducts can also alter the phenotypic characteristics of dental pulp stem cells, affecting the regenerative potential of the pulp tissue [11]. Depletion of glutathione, production of reactive oxygen species, and a few other molecular mechanisms were also identified as determining factors leading to apoptosis and/or pulp necrosis [12]. The evaluation of the biological effects resulting from the application of self-adhesive cements to recently exposed dentin, especially in total crown preparations is of clinical relevance and should not be neglected [13].

The cytotoxic effects of exposed odontoblast cells to commercial self-adhesive cements polymerized using different activation modes was previously evaluated [14]. All of the self-adhesive cements tested induced a significant decrease in the viability of MDPC-23 cells, affecting the cytotoxicity, total cell death, and type of cell death in diverse magnitudes. Thus, the concentrations of biologically active molecules released by the self-adhesive cements and their chemical formulation were high enough to modify the pulp metabolism of odontoblastic cells [14].

Considering that the exposure of an odontoblastic cell line to different self-adhesive cements may induce cytotoxicity to varied extents, we hypothesized that these materials may interfere in the oxidative stress response, the gene expression of different signaling pathways of the inflammatory process and transcriptional activators, and the expression of interstitial collagenases. Furthermore, we hypothesized that these alterations would ultimately lead to modifications in the phenotypic characteristics in terms of cellular proliferation and mineral deposition in MDPC-23 odontoblast-like cells.

2. Materials and methods

2.1. Experimental design

This in vitro study evaluated the oxidative stress responses, the signaling pathways of the inflammatory process/transcriptional activators, and the expression of interstitial collagenases in MDPC-23 odontoblast-like cells after exposure to self-adhesive cements: RelyX U200 (3M ESPE) and MaxCem Elite (Kerr) (Table 1). The phenotypic characteristics of cellular proliferation and mineralization potential were also evaluated.

2.2. Specimen preparation

The unpolymerized materials were then placed into a Teflon mold (2 mm thick, 6 mm in diameter) and positioned over a polyester strip. After filling the mold, the cement surface was covered with a Mylar strip and a glass slide to compress and extrude the excess material. The glass slide was then removed, leaving the Mylar strip. Specimens were then photoactivated using an LED light-curing unit (1200 mW/cm^2, Bluephase, Ivoclar Vivadent, Schaan, Liechtenstein) following the manufacturers' instructions. The specimens were removed from the moulds and stored in lightproof containers for 24 h at room temperature (22 °C).

Table 1 – Self-adhesive resin cementscharacterized in the present study[a].

Material	Lot no/exp. date	Composition	Working time (min)	Setting time (min)	Curing time (s)	Filler content wt (%) vol (%)
RelyX U200 3 M ESPE, St. Paul, MN, USA	659007 2018-08	Base: methacrylate monomers containing phosphoric acid groups, methacrylate monomers, initiators, stabilizers, rheological additives. Catalyst: methacrylate monomers, alkaline fillers, silanated fillers, initiator components, stabilizers, pigments, rheological additives. Zirconia/silica fillers. Clicker delivery system.	2	6	20	72.0 43.0
MaxCem Elite Kerr Corporation, Orange, CA, USA	6088085 2018-02	GPDM, co-monomers (mono-, di-, and tri-functional), proprietary self-curing redox activator, methacrylate monomers, water, acetone, and ethanol. Inert minerals and ytterbium fluoride. Automix system.	1	4	10–20	69.9 46.0

Abbreviations: GPDM: glycero-phosphate dimethacrylate.
[a] Manufacturers' information.

2.3. *MDPC-23 cell model*

MDPC-23 cells are derived from the 18-day-old CD-1 foetal mouse mandibular first molar papilla which synthesizes the dentine-specific protein DSP, which is commonly regarded as being of odontoblast lineage [15]. MDPC-23 cells are pre-odontoblast-like cells and possess odontoblast characteristics such as expressing dentine sialophosphoprotein (DSPP) and dentine matrix acidic phosphoprotein 1 (DMP-1) [16]. MDPC-23 cell line is considered a good cell model to study the possibility of generating odontoblasts directly from dental pulp/papilla cells [16]. MDPC-23 rat odontoblast-like cells were subcultured in Dulbecco's Modified Eagle's Medium (DMEM; Sigma Aldrich Corp., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Cultilab, Campinas, SP, Brazil), 100 IU/mL penicillin, 100 mg/mL streptomycin, and 2 mmol/L glutamine (Gibco, Grand Island, NY, USA) in a humidified incubator with 5% CO_2 and 95% air at 37 °C (Isotemp Fisher Scientific, Pittsburgh, PA, USA) for 3 days. MDPC-23 cells were counted with the aid of a Neubauer chamber, 0.100 mm depth, 0.0025 mm^2. About 3.8×10^4 cells were seeded in the lower compartment of 24-well plates (HTS Transwell, Ref. #3374, Corning, NY, USA) and maintained in an incubator with 5% CO_2 and 95% air at 37 °C.

2.4. *Determination of reactive oxygen species (ROS) production*

The production of ROS in MDPC-23 cells was determined with the fluorogenic probe H_2DCFDA (Invitrogen, Carlsbad, CA, USA). Fluorescence was monitored by confocal microscopy on a Carl Zeiss LSM 510 META instrument (Oberkochen, Germany). Cells were grown in 35-mm diameter culture plates for 48 h at a density of 10,000 cells/well and kept under maintenance conditions. Cells were incubated with cement specimens for 4 h to analyze the concentrations of ROS triggered by the cements. Then, cells were washed with PBS and incubated with 5 μM of H_2DCFDA (0.5% DMSO) in Tyrode's solution for 30 min at 37 °C. After the incubation time elapsed, cells were washed with culture medium and maintained for experimentation in Tyrode's solution (136.8 mM NaCl, 2.68 mM KCl, 1.36 mM $CaCl_2 \cdot 2H_2O$, 0.48 mM $MgCl_2 \cdot 6H_2O$, 0.36 mM $NaH_2PO_4 \cdot H_2O$, 12 mM $NaHCO_3$, and 5.55 mM glucose). The images were analyzed using Examiner software (Carl Zeiss, Oberkochen, Germany) and Spectralyzer, which determines the temporal analysis of regions in a chart.

2.5. *Quantification of gene expression*

Total RNA from MDPC-23 cells was extracted using the TRIzol reagent, and the reverse-transcribed complementary DNA (cDNA) was obtained using the ImProm-II Reverse Transcriptase System kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Real-time quantitative polymerase chain reaction (qRT-PCR) assays were performed using the SYBR Green PCR Master Mix (Thermo Fisher Scientific). The cycling parameters for the PCRs were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min in an ABI PRISM 7500 Real Time PCR System (Applied Biosystems). Relative quantification was performed using the

ΔCt method which results in ratios between target genes and a housekeeping reference gene, the enzyme β-2-microglobulin for MDPC-23 cells (control). The primers for the different rat genes studied were manufactured by Exxtend (São Paulo, SP, Brazil). The specificity of the amplification reaction was determined with a melting curve analysis.

2.6. Cell proliferation assays

MDPC-23 cells were seeded at 3×10^4 cells/well in 24-well plates and grown to 70–80% confluence. Cells were then starved in serum-free medium overnight and maintained in minimal essential medium without serum overnight. Then, cells were incubated with resin cement discs for 4 h or DMEM high glucose for 4 h at 37 °C. FBS was added to the plate at various concentrations (0.1, 1.0, and 10%), and cells were incubated for 24 h. Finally, 10 μM BrdU was added to the plate, and cells were incubated for 4 h as determined by the BrdU Cell Proliferation Assay Kit (Cell Signaling Technology).

2.7. Alizarine red staining mineralization assay

The Alizarin Red S assay was used to determine the influence of U200 and MAX on the degree of hydroxyapatite mineralization in MDPC-23 cell culture. Cells were seeded in 24-well plates at a density of 3×10^4 per well and cultured in DMEM high-glucose containing 10% (v/v) FBS. After 24 h of incubation, the cells were incubated with resin cement discs for 4 h in the mineralization media containing DMEM high-glucose media, 10% FBS, 50 μg/mL ascorbic acid, and 5 mM β-glicerophosphate. Controls were treated with the mineralization solution in the absence of resin cement pretreatment. Cell cultures at days 8, 16, and 24 were washed with PBS, fixed with 4% (v/v) formalin for 15 min at room temperature, washed twice with distilled water, then stained with 40 mM Alizarin Red S (pH 4.1) for 30 min at 25 °C with gentle agitation. To observe the alizarin staining in MDPC-23 cultures, the plates were analyzed using the inverted light microscope Observer A1 Axio (Carl Zeiss, Oberkochen, Germany). After staining, cultures were washed three times with distilled water; then 10% (v/v) acetic acid was added to each well for 30 min at 25 °C for destaining, followed by 10% ammonium hydroxide to neutralize the acid. The intensity of the red color formed was measured at 510 nm using a microplate reader. Data are expressed as absorbance units at 510 nm of Alizarin Red S released per cell culture.

2.8. Statistical analysis

Statistical significance among the groups was assessed using one-way analysis of variance (ANOVA), followed by Tukey's test. The unpaired two-tailed Student's t test ($P < 0.05$) was applied to test for significant differences between the two self-adhesive cements (GraphPad Prism software, CA, USA).

3. Results

Cements U200 and MAX induced oxidative stress in the MDPC-23 cells in different ways (Fig. 1). MAX promoted significantly higher oxidative stress in the cell culture compared to that when exposed to U200. The latter induced a small but significant increase in the basal fluorescence leading to an increased production of ROS (1.4-fold) compared to control group ($P = 0.0094$). However, MAX induced a stronger oxidative stress, significantly increasing ROS production (2.8-fold) when compared to the control group ($P = 0.0004$). As expected, the positive control H_2O_2 (100 μM) promoted a large increase in ROS production (16-fold) when compared to the control group ($P < 0.0001$).

Keap1–Nrf2–ARE signalling controls genes encoding phase 2 detoxifying enzymes and antioxidant proteins [17]. To examine the effects of both resins on the expression of antioxidant genes that are downstream of Nrf2, mRNA expression was also analysed by using real-time PCR (Fig. 2G and H). As indicated by mRNA level, U200 exposure for 4 h only increased the expression of *thioredoxin reductase* 1 (TRXR1) and *heme oxygenase* 1 (HO-1) antioxidant genes in MDPC-23 cells. Except for TRXR1 and HO-1, which were increased by two-fold and six-fold, respectively, as compared with control ($P < 0.05$), the treatment with the U200 resin did not change the expression of the remainder antioxidant genes tested in this study, namely *glutaredoxin* 1 (GRX1), *thioredoxin* 1 (TRX1), *glutathione peroxidase* 1 (Gpx1), *cytoplasmic superoxide dismutase* (SOD1), and catalase (CAT). These results indicate that U200 treatment can induce only a moderate increase in antioxidant genes expression. As expected, MAX exposure significantly increased the mRNA expression of more antioxidant genes than U200 did, including TRXR1, HO-1, SOD1, and CAT in MDPC-23 cells ($P < 0.05$). Among the antioxidant genes that are downstream of Nrf2, the expression HO-1, TRXR1, SOD1, and CAT was increased by four-, three-, 1.7-, and 1.6-fold, respectively, in MAX-exposed cells as compared with control.

Differences in the intensity of the oxidative stress induced by these cements seem to explain the variation observed in the expression of the studied genes. By exposing the MDPC-23 cells to both cements for 4 h under experimental conditions, different gene expression responses were observed (Fig. 3). MAX induced an increased expression of: *dentin sialophosphoprotein* (DSPP) (4-fold), *metalloproteinase* (MMP)-1 (11-fold), *tumor necrosis factor* (TNF)-α (6-fold), *peroxisome proliferator-activated receptor-gamma coactivator 1 alpha* (PGC-1α) (8-fold), *peroxisome proliferator-activated receptor gamma* (PPAR-γ) (3.5-fold), and *un-coupling proteins* (UCPs) (7-fold). The expression of the *phosphate-regulating endopeptidase homolog X-linked* (PHEX) and *nuclear factor-kappa B* (NF-kB) genes was also slightly but significantly induced by MAX (1.6-fold in both cases). Conversely, U200 induced the expression of *dentin matrix protein* 1 (DMP-1) (3-fold), *specificity protein* 7 (Sp7) (6-fold), *runt-related transcription factor* 2 (Runx2) (10-fold), MMP-2 (1.7-fold), and *nuclear factor (erythroid-derived 2)-like-2 factor* Nrf2 (2-fold).

The changes in the gene expression balance induced by the cements were also proven by the phenotypic analyses of the cellular proliferation disturbance (Fig. 4) and by the cell mineralization potential (Fig. 5). MAX significantly inhibited cell proliferation in 0.1 and 1.0% FBS. In 10% FBS, there was a slight but not significant inhibition of cell proliferation induced by MAX. Conversely, U200 significantly stimulated cell proliferation (around 30%), irrespective of the concentration of FBS. MAX delayed the mineralization potential, especially at ini-

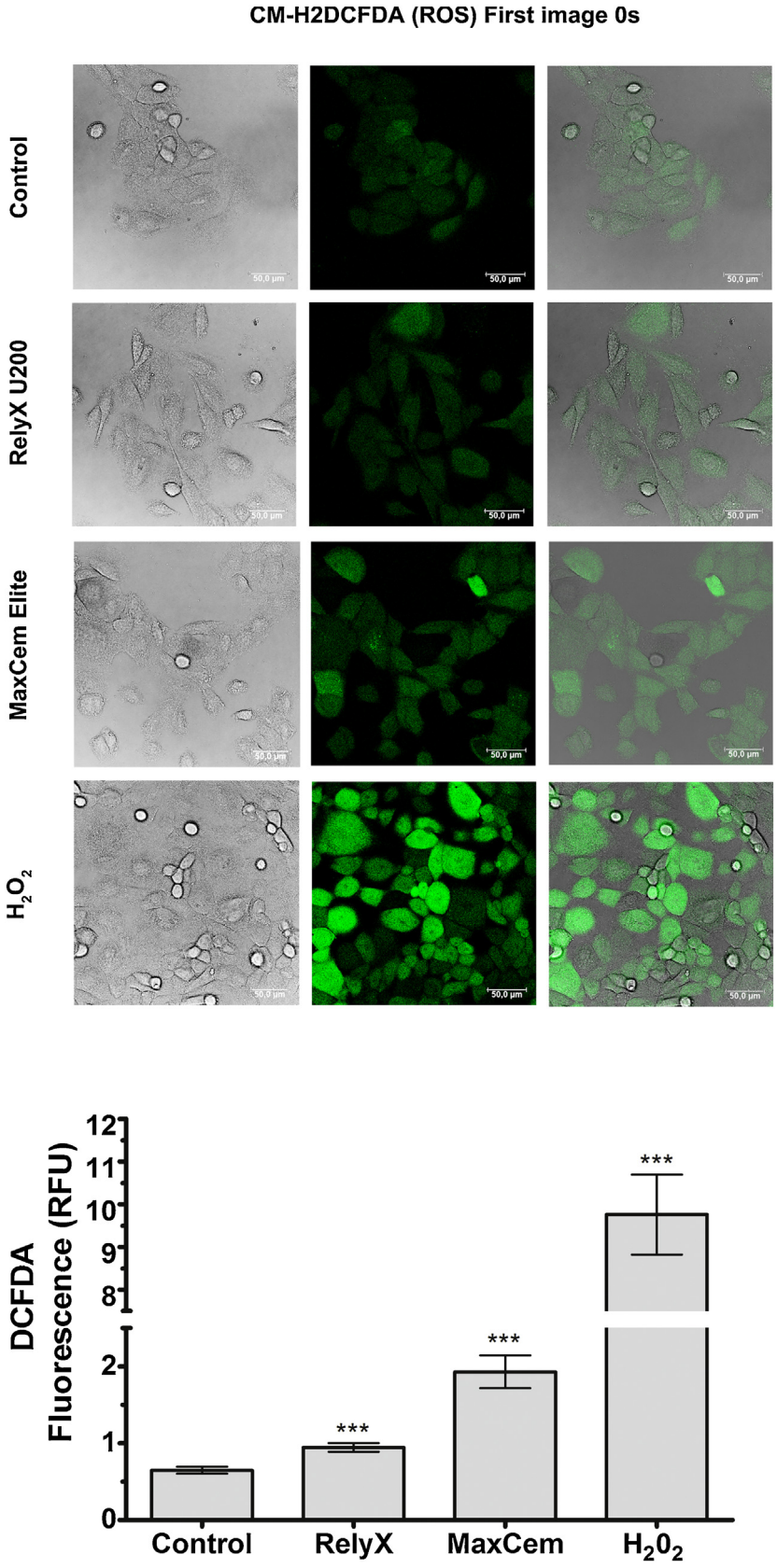

Fig. 1 – ROS generation in MDPC-23 cell line. Representative fluorescent microscopy image of MPDC-23 cells exposed to cement specimens obtained by confocal microscopy. The presence of ROS in MDPC-23 cells was monitored by measuring the green fluorescence emitted by H2DCFDA after 30 min of probe incorporation. Scale bars, 50 μm. The bar graph represents the mean and standard error of the fluorescence intensity measurements of MDPC-23 cells exposed to specimens of MaxCem Elite, RelyX U200 or 100 μM H_2O_2 for 4 h in comparison with the control, unexposed cells (N = 10).

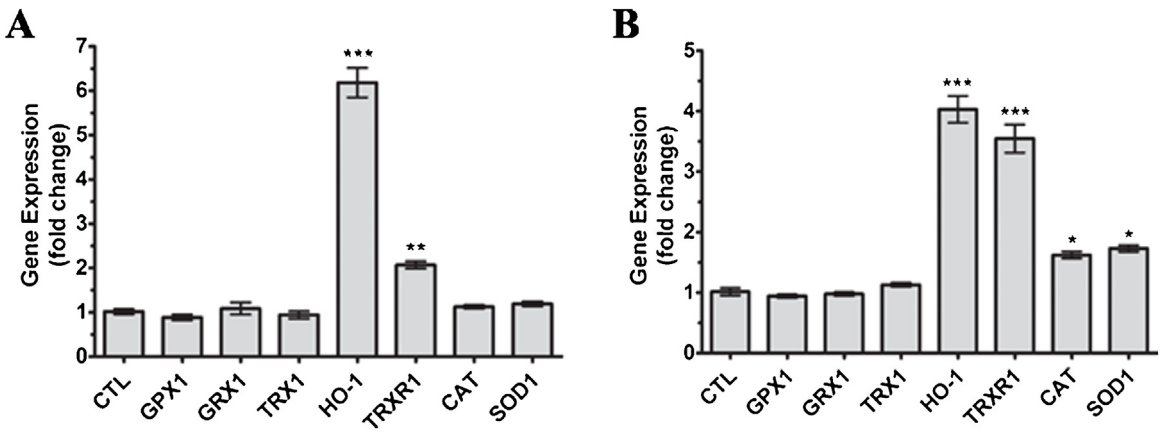

Fig. 2 – RelyX U200 (A) and MaxCem Elite (B) exposure induce the expression of Nrf2 downstream antioxidant genes. Real-time quantitative PCR (qRT-PCR) analysis of gene expression in odontoblast-like MDPC-23 cells were exposed for 4 h to cement specimens of RelyX U200 and MaxCem Elite, respectively. The bars represent the expression levels of genes involved in antioxidant genes that are downstream of Nrf2 (A and B): thioredoxin reductase 1 (TRXR1), heme oxygenase 1 (HO-1), glutaredoxin 1 (GRX1), thioredoxin 1 (TRX1), glutathione peroxidase 1 (Gpx1) cytoplasmic superoxide dismutase (SOD1) and catalase (CAT). The qRT-PCR analysis of gene expressions was normalized to the housekeeping gene enzyme β-2-microglobulin for MDPC-23 cells (control) (mean ± SEM); the relative quantification of the expression levels (experimental/control) was determined based on the 2-[Δ]Ct method as described previously. All experiments were performed in triplicate. Asterisk indicate statistically significant differences ($P < 0.05$).

tial times (8th and 16th days), in which mineral deposition is reduced as indicated by the intensity of alizarin red; only at the 24th day was the mineralization process re-established after exposure to MAX (Fig. 5).

4. Discussion

Odontoblasts through inflammatory stimuli are able to orchestrate the inflammatory response in the dental tissues by producing and secreting a wide variety of cytokines and chemokines [18,19]. The exposition of the odontoblast cells to both cements triggered the expression of antioxidant genes that are downstream of Nrf2 against the released toxic by-products from these cements.

The major adaptive cell response to induced oxidative stress relies on the cross-talk and interdependence between various Nrf2-regulated antioxidant pathways [20]. Nrf-2 enhances the structural and functional integrity of mitochondria under stress conditions via relationship with various proteins, including TRXR1, GRX1, TRX1, GPX1, SOD1 and CAT [21]. Nrf-2 is also a potent HO-1 inducer, a stress-response protein that also plays an important role in the signal transduction pathways used to detect adverse environmental conditions [22]. HO-1 is also an important cellular target for odontoblastic differentiation in dental pulp cells [23]. The exposition of the odontoblast cells to both cements triggered cytoprotective genes mediated by the upregulated expression of HO-1 against the released toxic byproducts from these cements. Mediated via Nrf2, HO-1 expression was significantly induced by U200 (6-fold) and MAX (4-fold) (Fig. 2). Induced Nrf2 expression (2-fold) somehow explains the higher HO-1 expression when exposed to U200 (Fig. 2).

Expression of HO-1 has been claimed to inhibit the maturation in osteoblasts, including mineralized bone nodule formation, *alkaline phosphatase* (ALP) activity, and decreased mRNA expression of differentiation markers such as ALP, *osteocalcin* (OCN), and Runx2 [24]. Runx2 is a transcription factor that takes part in odontoblast differentiation [25], which is followed by reparative dentin formation [26]. Runx2 and its downstream target, Sp7, are essential transcription factors for development of the mineralizing cell types [27]. U200 significantly induced the overexpression of Runx2 (10-fold) and Sp7 (6-fold), whereas Runx2 gene expression was significantly downregulated by MAX (Fig. 3). Sp7 remained unaltered in the presence of MAX.

Mitochondrial biogenesis can be activated by oxidative and inflammatory cell stress. However, higher levels of oxidative stress trigger inflammatory mitochondrial dysfunction, resulting in energetic failure and cell death [28]. Oxidative and nitrosative stress stimulate mitochondrial biogenesis via the induction of the nuclear transcription factor PGC-1a coactivator [28]. In a previous study, MAX was shown to be more aggressive to cells in comparison to other cements [14]. Increased ROS production after exposure to MAX extracts has also been regarded as the main reason for its higher cytotoxicity [13]. MAX caused the most intense oxidative stress in MDPC-23 cells which was statistically significantly different compared to that of the negative control group (Fig. 1). Higher levels of oxidative stress induced by MAX can upregulate the expression of early-phase inflammatory protein mediators such as TNF-α (Fig. 3). The TNF-α is one of the most potent physiological inducers of the nuclear transcription factor NF-Kb [29]. TNF-α receptor operating through NF-κB can promote upregulation of PGC-1α [30].

Indeed, the MDPC-23 cells overexpressed TNFα and PGC-1α in the presence of MAX (Fig. 3). As mentioned above, PGC-

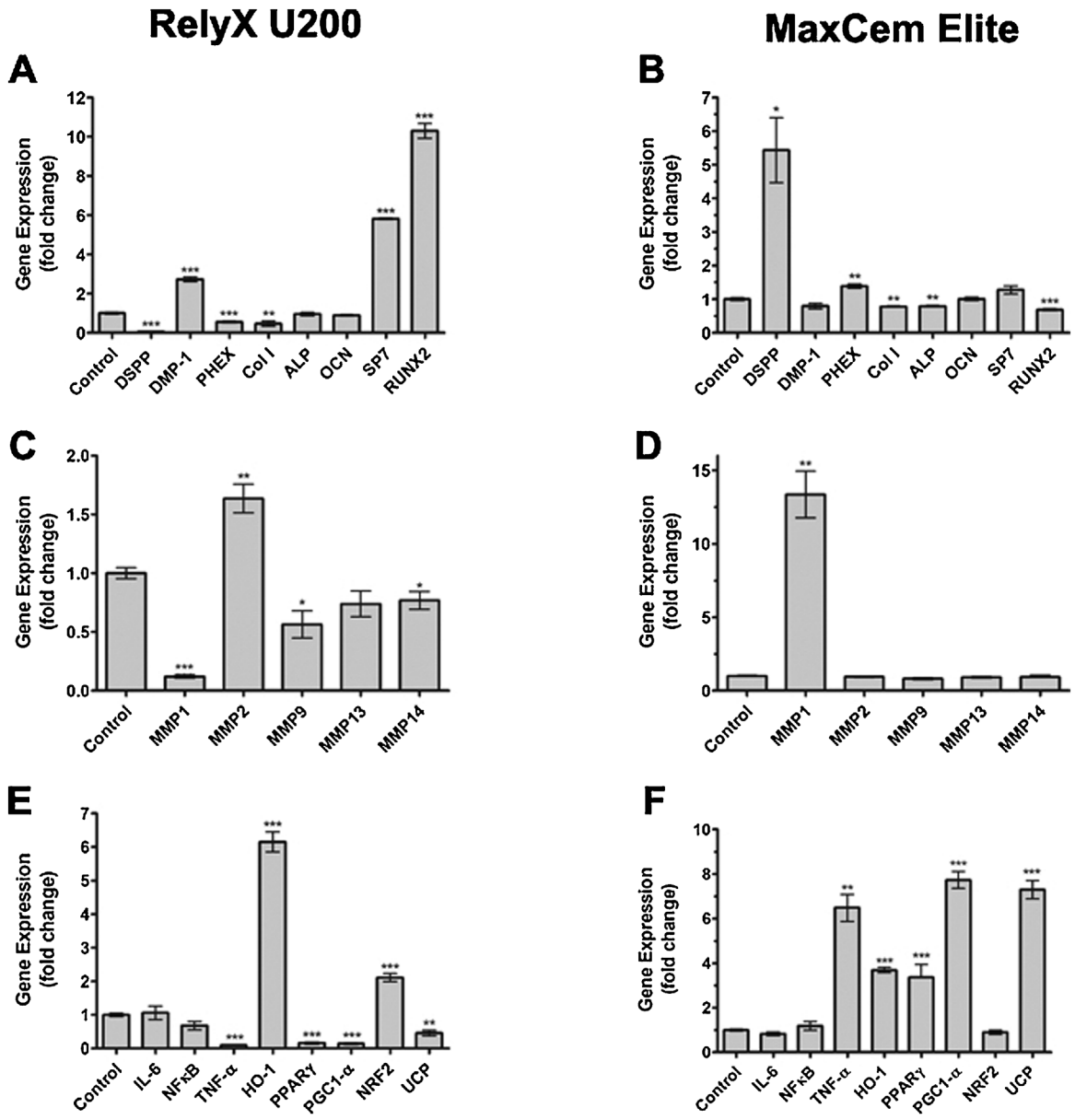

Fig. 3 – Real-time quantitative PCR (qRT-PCR) analysis of gene expression in odontoblast-like MDPC-23 cells were exposed for 4 h to cement specimens of RelyX U200 and MaxCem Elite, respectively. The bars represent the expression levels of genes involved in the mineralization process (A and B): runt-related transcription factor 2 (Runx2), specificity protein 7 (Sp7), osteocalcin (OCN), collagen type-I (COL-I), alkaline phosphatase (ALP), X-chromosome (PHEX), dentin matrix protein 1 (DMP-1), and dentin sialophosphoprotein (DSPP); in the degradation of the extracellular matrix (C and D): matrix metalloproteinases (MMP)-1, MMP-2, MMP-9, MMP-13, and MMP-14, in the oxidative stress/inflammation process (E and F): Un-coupling Proteins (UCP), nuclear factor (erythroid-derived 2)-like-2 factor (Nrf2), peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1α), peroxisome proliferator-activated receptor gamma (PPAR-γ), heme oxygenase-1 (HO-1), tumor necrosis factor (TNF)-α, nuclear factor-kappa B (NF-κB), and interleukin (IL)–6. The qRT-PCR analysis of gene expressions was normalized to the housekeeping gene enzyme β-2-microglobulin for MDPC-23 cells (control) (mean ± SEM); the relative quantification of the expression levels (experimental/control) was determined based on the 2-[Δ]Ct method as described previously. All experiments were performed in triplicate. Asterisk indicate statistically significant differences ($P < 0.05$).

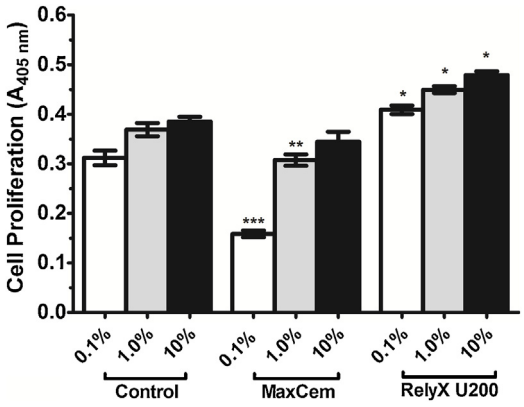

Fig. 4 – Effect of the cements RelyX U200 and MaxCem Elite on cellular proliferation of MDPC-23 cells after 4-h exposure as a function of the fetal bovine serum concentration. The bars represent the mean ± standard deviation (N = 3). Statistical analysis was performed with ANOVA test followed by Bonferroni. Cell proliferation of MDPC-23 cells was analyzed at three different concentrations of fetal bovine serum (FBS, concentrations 0.1%, 1.0%, and 10%) by the BrdU assay, according to the manufacturer's instructions.

1α is a major regulator of mitochondrial biogenesis. Also, PGC-1α modulates anti-inflammatory [31] and antioxidant pathways [32]. Increasing PGC-1α expression results in induction of several mitochondrial biogenesis genes, including the upregulation of UCP-1 (Fig. 2). Additionally, MAX oxidative stress can also induce the HO-1/CO system (Fig. 3). HO-1/CO system stimulates both mitochondrial biogenesis and antioxidant genes [28]. Taken together, our results strongly suggest that MAX oxidative stress upregulates TNF-α expression, an early-phase inflammatory mediator protein, that can interact with its receptor to activate NF-κB pathways, resulting in increased expression of the transcription factor PGC-1α which in turn regulates mitochondrial biogenesis and antioxidant gene expression.

TNF-α is also a major cytokine that plays to a negative role in bone metabolism in many inflammatory diseases or pathological processes [33]. TNF-α promotes bone loss by inhibiting osteoblastic differentiation and bone formation [34]. Runx2 has an essential role in osteo/odontoblastic differentiation of stem cells [35] and directly stimulates transcription of its important downstream target genes, including those encoding ALP, OCN and *collagen type-I* (COL-1) [34]. TNF-α inhibits osteoblast differentiation through suppression of Runx2 expression [36]. We found that the exposure of the MDPC-23 cells to MAX caused a large expression of TNF-α with a concomitant large decrease in the expression of Runx2 and its downstream target genes ALP and COL-1 (Fig. 3). Moreover, TNF-α can also be related to the upregulation of MMP-1 gene [37] in presence of MAX (ss. 3). Consistent with the effects observed for the early odontogenesis markers Runx2, ALP, and COL-1; alizarin red staining and quantification were also severely decreased in the MaxCem-treated MDPC-23 cells (Fig. 4). Additionally, MAX-treated MDPC-23 cells promoted a large decrease in cellular proliferation stimulated by FBS (Fig. 4). Taken together, our results strongly indicate that MaxCem treatment causes high levels of oxidative stress in MDPC-23 cells inducing upregulation of TNF-α expression, a major inflammatory cytokine, that triggers inflammatory mitochondrial dysfunction with a concomitant reduction of the expression of Runx2. The inhibition of Runx2 expression caused a large decrease in odontoblastic-like MDPC-23 cell proliferation and differentiation.

Conversely, U200 induces a small oxidative stress in MDPC-23 cells when compared to MAX (Fig. 1). The lowest (mild) intensity oxidative stress is mainly sensed by the Nrf2/Keap1 system. Thus, under oxidative stress, Nrf2 dissociates from Keap1, thus translocating to the nucleus and activates the expression of antioxidant response element (ARE)-genes. Indeed, only U200 was able to upregulate the expression of the Nrf2 gene (Fig. 3). In turn, the elevation of Nrf2 expression leads to increased expression of the HO-1, which is one of the classic Nrf2-regulated genes. Interestingly, Nrf2/HO-1 axis upregulation plays a major role in anti-inflammatory function [38]. Therefore, the observed decrease in expression of the U200-induced NF-κB and TNF-α inflammatory genes in MDPC-23 cells can be attributed to upregulation of the Nrf2/HO-1 axis [39]. In inflammation, it has been reported that the Nrf2/HO-1 axis also decreases the expression of several MMPs directly by the Nrf2 pathway or indirectly through the Nrf2-influenced NF-κB pathway. Indeed, in the presence of U200, the levels of expression of MMP-1, MMP-9, and MMP-14 significantly decreased (Fig. 3).

U200 can delay the osteogenic differentiation of MDPC-23 cells (Fig. 5) by decreasing expression of COL-I, PHEX, and DSSP genes and activating the expression of DMP-1, a member of the SIBLING family of extracellular matrix proteins that is able to inhibit the hydroxyapatite crystal formation and growth [40]. The expression of these osteogenic genes is strongly regulated by the NF-κB signalling pathway via targeting TNF-α [41]. During the inflammation, the activation of NF-κB can inhibit the capacity of odontoblast differentiation. However, autophagy decreases the nucleus translocation of p-NF-κB by its degradation, enhancing the odontoblast differentiation capacity. The expression of DMP-1 and Runx2/SP7 (OSX) genes was upregulated when autophagy was enhanced and p-NF-κB was inhibited [42]. U200 induced the upregulated expression of DMP-1 and Runx2/SP7 as expected (Fig. 3). Also, upregulation of mitochondrial biogenesis has been shown to be involved during osteogenic differentiation [43]. The mRNA expression level of UCP, PGC-1α, and PPARγ was also severely decreased in the presence of U200 (Fig. 3). UCP, regarded to provide protection from oxidative damage by preventing excessive production of mitochondrial ROS [44], directly controls the alterations of ATP synthesis and ROS generation [45]; PGC-α1 and PPARγ are major transcriptional factors required for mitochondrial biogenesis that are upregulated during osteogenic differentiation [43]. In general, U200 suppressed the osteogenic differentiation of MDPC-23 cells by blocking the NF-κB signalling pathway via upregulation of the Nrf2/HO-1 axis triggered by mild oxidative stress induced by this cement.

The Runx2/SP7 axis is detected in preosteoblasts, and the expression is upregulated in immature osteoblasts and downregulated in mature osteoblasts; decreasing levels of Runx2

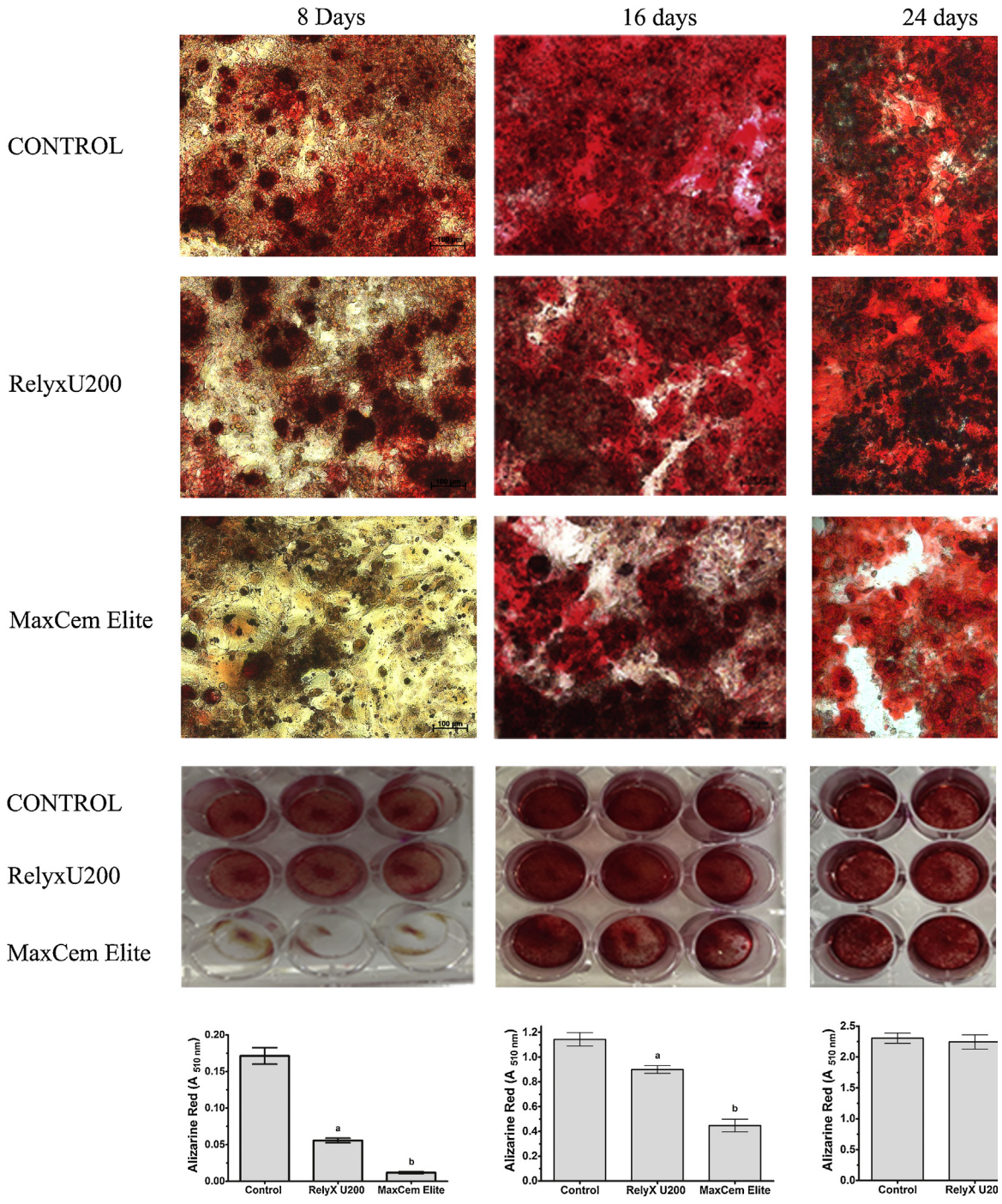

Fig. 5 – The alizarin red S assay was used to determine the influence of resin cements RelyX U200 and MaxCem Elite on the degree of hydroxyapatite mineralization in MDPC-23 cell culture. The quantification of the hydroxyapatite formed in MDPC-23 cells by alizarin staining was monitored at 8, 16, and 24 days of mineralization after exposure to cement specimens of RelyX U200 and MaxCem Elite. The intensity of the red color formed was measured at 510 nm using a microplate reader. The difference in color intensity between treated cells and control, unexposed group after alizarin red stain indicates reduced mineral formation after cement exposure. Data expressed as absorbance units at 510 nm of Alizarin Red S released per cell in each culture. The bars represent the average ± standard error (N = 3). Lowercase letters indicate the statistical significance ($P < 0.05$) as measured by the unpaired t test.

lead to cell cycle exit for proliferating and differentiating osteoblasts [46]. U200 increased the proliferation of MDPC-23 cells independently of the concentration of FBS (Fig. 5). The inhibition of the NF-κB signalling pathway triggered by U200 can also upregulate the Runx2/SP7 axis in the MDPC-23 cell cycle, favouring its cellular proliferation.

5. Conclusions

Biologically active components released by both cements were high enough to modify the odontoblast cell metabolism in different ways. Gene expression disturbance induced by the cements was confirmed by the phenotypic analyses in terms of cellular proliferation and mineralization potential. These results highlight the need for the development of newly bioactive dental restorative materials considering the problems related to the clinical application of functional acidic-containing cements with respect to harmful effects in odontoblastic cells, which might delay the reparative dentin formation after cementation of indirect restorations, especially on recently exposed dentin preparations.

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