ORIGINAL ARTICLE

Response of non-differentiated pulp cells (OD-21) to a novel bioceramic for dental pulp capping

ABSTRACT

Aim: *To evaluate an experimental bioceramic (QCP5) effect on undifferentiated pulp cells (OD-21 cell lineage).*
Methodology: *Cells were divided into three groups: control (untreated cells), QCP5 (cells in indirect contact with biomimetic ceramic (QCP5), and Mineral Trioxide Aggregate (cells in indirect contact with [MTA]). Cell viability, biochemical ALP activity, fast red* in situ, *and mineralization were evaluated. The proteins Alp, Col1A1 and DMP1 were detected by immunofluorescence. The data were analyzed by a variance analysis with 5% significance level.*
Results: *The cell viability test at 3 and 7 days was similar between the groups, but at 10 days there was a significant increase in both the MTA and the QCP5 groups. It was observed that at 3 and 10 days there was no significant difference in ALP activity, but at 7 days the expression of ALP was greater in the control group compared with QCP5 and MTA exposure groups. The fast red technique at 3 and 10 days showed no significant difference, but at 7 days it was significantly lower for the control group compared with QCP5 (P = 0.0477) and MTA (P = 0.0217). Mineralization was significantly higher in the control group (P = ≤0.0001). ALP protein marking was similar in all three groups, Col1a1 presence was significantly decreased in the MTA group, and DMP1 was more marked in the QCP5 group.*
Conclusion: *The bioceramic QCP5 promoted cell viability, expression of ALP, mineralization and expression of proteins ALP, Col1a1, and Dmp1. Similar behavior was observed with cells exposed to MTA.*

Lina C. González-Pita[1]

Paula Katherine Vargas-Sanchez[2]

Karina Fittipaldi Bombonato-Prado[2]

Edgar Delgado-Mejía[3]

Carolina Torres-Rodríguez[1*]

[1]Faculty of Dentistry, National University of Colombia-Sede Bogotá, Colombia

[2]Bone Research Lab, Department of Basic and Oral Biology, School of Dentistry, University of São Paulo, Ribeirão Preto, SP, Brazil

[3]Department of Chemistry, Faculty of Sciences, National University of Colombia-Sede Bogotá, Colombia

Received 2023, January 29

Accepted 2023, March 3

KEYWORDS *biocompatible materials, biomineralization, calcium phosphates, dental pulp capping, MTA cement.*

Corresponding Author
Carolina Torres Rodriguez | Faculty of Dentistry, Universidad Nacional de Colombia-Sede Bogotá | Colombia
Email: ctorresr@unal.edu.co Phone: 6013165000 ext 16017

Peer review under responsibility of Società Italiana di Endodonzia

10.32067/GIE.2023.37.01.12

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Introduction

Vital pulp therapy (VPT) has been proposed to preserve and promote integrity of the mineralized tissue barrier of the pulp vitality of deciduous or young permanent teeth with immature roots affected by caries and without evidence of radicular pathology. Nowadays, treatment options of VPT are represented by indirect pulp treatment (namely indirect pulp capping), direct pulp capping and pulpotomy. In addition, the success of this technique would avoid pulpectomy and subsequent root canal obturation by several materials, that, in turn, could prevent the radicular resorption of the primary molars and alter the development of the permanent teeth (1).

Properties like cytocompatibility are expected from the biomaterials used for such purpose, meaning that when placed in direct contact with the cellular component of vital tissues, these will express physiological levels of proliferation, migration, and survival. In addition, the materials used in VPT should exhibit bioactive properties, i.e., induce the formation of a mineralized hydroxyapatite-like attachment to the dentine substrate through the ionic interchange with tissue fluids in the process of biomineralization. Lastly, biomaterials placed in contact with the dentin–pulp complex should ideally influence cell plasticity, inducing the osteo/odontogenic differentiation of local human dental pulp stem cells (HDPSCs) and, consequently, promote the process of tissue repair upon damage (2).

The most frequently used agents for VTP are calcium hydroxide (CH), di- and tricalcium silicates (mineral trioxide aggregate (MTA), Biodentine (BD) (1) and calcium phosphates with silicon dioxide, tricalcium phosphate (TCP) Cement®, Bioglass® or phosphates without silica. CH was one of the first materials with bioactive characteristics (introduced in the 1920s) used to promote the formation of a dentinal bridge on exposed pulp tissue (3), although CH has been successfully used for its biological and antimicrobial properties, its disadvantages include low mechanical properties, cytotoxicity, poor sealing, lack of adhesion to substrate, microfiltration and high solubility (4-9). The above mentioned materials arose as an alternative to CH because it is a biocompatible material with antibacterial properties, excellent sealing over time, and formation of a thick and homogeneous dentinal bridge that is not reabsorbed. However, these cementitious materials also exhibit several drawbacks such as being difficult to manipulate, long setting time, high cost, difficult storage, uncorrectable discoloring effect associated to the iron ion, and their cytotoxicity by the presence of metallic ions such as Al^{+3} y $Bi^{+3,+5}$ (5, 7, 10-15).

Currently, ceramic materials based on calcium phosphates are popular among dental practitioners because they are biomimetic, biocompatible, bioinductive, and have better physical and mechanical properties than MTA. Bioceramics have a composition similar to the human mineral component (16), and they are able to stimulate components of the extracellular matrix and induce odontoblastic differentiation (17) and hydroxyapatite formation (5). QCP5 is a ceramic material made from eggshell comprising several phases, and several alkaline phosphates, like apatites and tricalcium phosphate (TCP) suitable for inducing rapid mineralization.

Recent advances in tissue and cell engineering have been contributing to repair injured tissues by using mesenchymal stem cells, which have been known to foster self-renewing properties (i.e., proliferation without a change in phenotype) and the capacity to differentiate into one or many different specialized cell types (i.e., changing into a new phenotype). In the Dentistry field, stem cells derived from dental pulp have been isolated and investigated as possible sources for regeneration of injured tissues. Immortalized murine pulp cells (OD-21) are undifferentiated mesenchymal stem cells derived from fetal molar papillae. It has been suggested that OD-21 lineage has potentiality to differentiate into odontoblast-like cells, under appropriate conditions, which might be a favorable source for pulp therapy (18).

The objective of our study was to evaluate the effect of the experimental biomimetic ceramic, QCP5, on OD-21 cells. The null hypothesis was that the QCP5 doesn't promote cell viability, alkaline phosphatase (ALP) expression, formation of mineralization nodules, or protein expression to reach a statistical significance greater than the MTA group.

Materials and Methods

This project was approved by the Ethics Committee B-CIEFO 178-17 of the School of Dentistry of the Universidad Nacional de Colombia-Sede Bogotá.

Preparation of QCP5

Calcium phosphate was biomimetically synthesized from chicken eggshells dried at 140°C in an oven without affecting the structure or stoichiometry and was then used to manufacture QCP5.

The dried eggshell material was fired in a furnace to obtain basic calcium oxide, which was then subjected to aqueous ortho-phosphoric acid solution to produce calcium phosphates. The detailed procedure and the instrumental characterization as well as the results are described in reference (19). The starting material and final product were controlled by X-ray diffraction (The X-ray diffraction (XRD) patterns were obtained using an X'Pert Pro MPD® (PANalytical) diffractometer with CuKα radiation (λ=1.5406 nm), produced at 40 mA and 45 kV. Scans were performed between 2θ values of 5º and 60º with an angular step of 0.0042º, and 5.08 s counting time per step); infrared spectra were obtained using a FT/IR Nicolet iS10 spectrometer (Thermo Fisher Scientific ®) and collected between 600 to 4000 cm^{-1} in absorbance mode, 124 scans at 1 cm^{-1} resolution) (20) and SEM-EDS data were obtained with a Tescan Vega 3 scanning electron microscope at 15,0 KV. The product was packed in transparent pharmaceutical glass vials, marked, sealed with pharmaceutical quality rubber stoppers and aluminum flip-off caps, and then exposed to 25 kGy of gamma radiation for 55 minutes for sterilization.

Preparation of materials

ProRoot MTA (Dentsply®) and QCP5 material were prepared in a laminar flow booth following the manufacturer's instructions.

Cell culture (OD-21 lineage)

The undifferentiated pulp cells, derived from the dental papilla of first molars of lab mice (OD-21 lineage) (21), were seeded by Prof. Jaques Eduardo Nor, of the School of Dentistry in the University of Michigan, USA. Cells were cultivated in 75 cm^3 vials with 10 mL of D-MEM culture medium, bovine fetal serum (10%), penicillin (100 UI/mL), streptomycin (100 µg/mL) and plasmocin (5 µg/mL). Then cells were cultured in 24-well plates at a density of 1×10^4 cells per well. Next, 50 µg of ascorbic acid was added to the culture medium, to favor the formation of collagen and extracellular matrix (22) and 2 mM of beta-glycerolphosphate was added last (23). The cells were immediately exposed to the materials using transwell inserts with 0.4 µm pores in the following groups:

-Control (-): OD-21 cells without material (n=5).

-QCP5: OD-21 cells with QCP5 (n=5).

-Control (+): MTA OD-cells with MTA (n=5).

Cell viability

After 3, 7, and 10 days of culture cell viability was evaluated by means of the MTT colorimetric assay ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] bromide) (Sigma). Aliquots of 150 µL were transferred to a plate of 96 wells for spectrophotometer reading (µQuant, Bio-tek Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm (24).

ALP activity

The detection of ALP at 3, 7, and 10 days was determined by the release of thymolphthalein by hydrolysis of the thymolphthalein monophosphate substrate, using the Labtest commercial kit (Diagnostica SA, Lagoa Santa, MG, Brazil) and following the manufacturer's instructions. Absorbance was determined in the spectrophotometer (Bio-Tek) with a wavelength of 590

nm. ALP activity data were normalized with total protein content.

In situ *ALP analysis by fast red*
The analysis was performed at 3, 7, and 10 days, after removing the culture medium the wells were washed twice with phosphate-capped saline (PBS) at 37ºC. A solution was prepared with trizma (Sigma-Aldrich), dimethylformamide (Merck), naphthol (Sigma-Aldrich) and nuclear fast red reagent (Sigma-Aldrich). 1 mL of this final solution was placed in each well for 30 min. When the time had expired, photographic documentation and quantitative analysis was performed with the Image J® program.

Detection and quantification of mineralized matrix
After 14 days, the wells were washed three times with PBS (Gibco) at 37 ºC. 2 mL of 10% formalin was added for fixation and then the solution was stored at 4 ºC for 24 hours. Formalin was removed from the wells and then dehydrated at room temperature with alcohols (30º, 50º, 70º, and 100º). After drying, the wells were stained with alizarin red 2%, pH 4.2 (Sigma). The protocol of Gregory et al. was followed to quantify mineralization nodules (25).

Immunofluorescence
After 5 days, the cells cultured in the wells were fixed in paraformaldehyde at 4%, pH 7.2. The cells were processed for indirect immunofluorescence. Three separate antibodies were used: monoclonal ALP antibodies (Sigma-Aldrich, USA) type I and Dmp1 collagen (Larry Fisher, USA), followed by secondary antibodies such as fluoride 594 for red fluorescence, and phalloidine 488 for green fluorescence (Molecular Probes, USA). The cells' nuclei were marked with 4,6-diamidine-2-phenylindole, dihydrochloride (DAPI, Molecular Probes). The images were examined under a fluorescence microscope attached to a camera (Leica, Germany).

Statistical analysis
The variance analysis was performed using

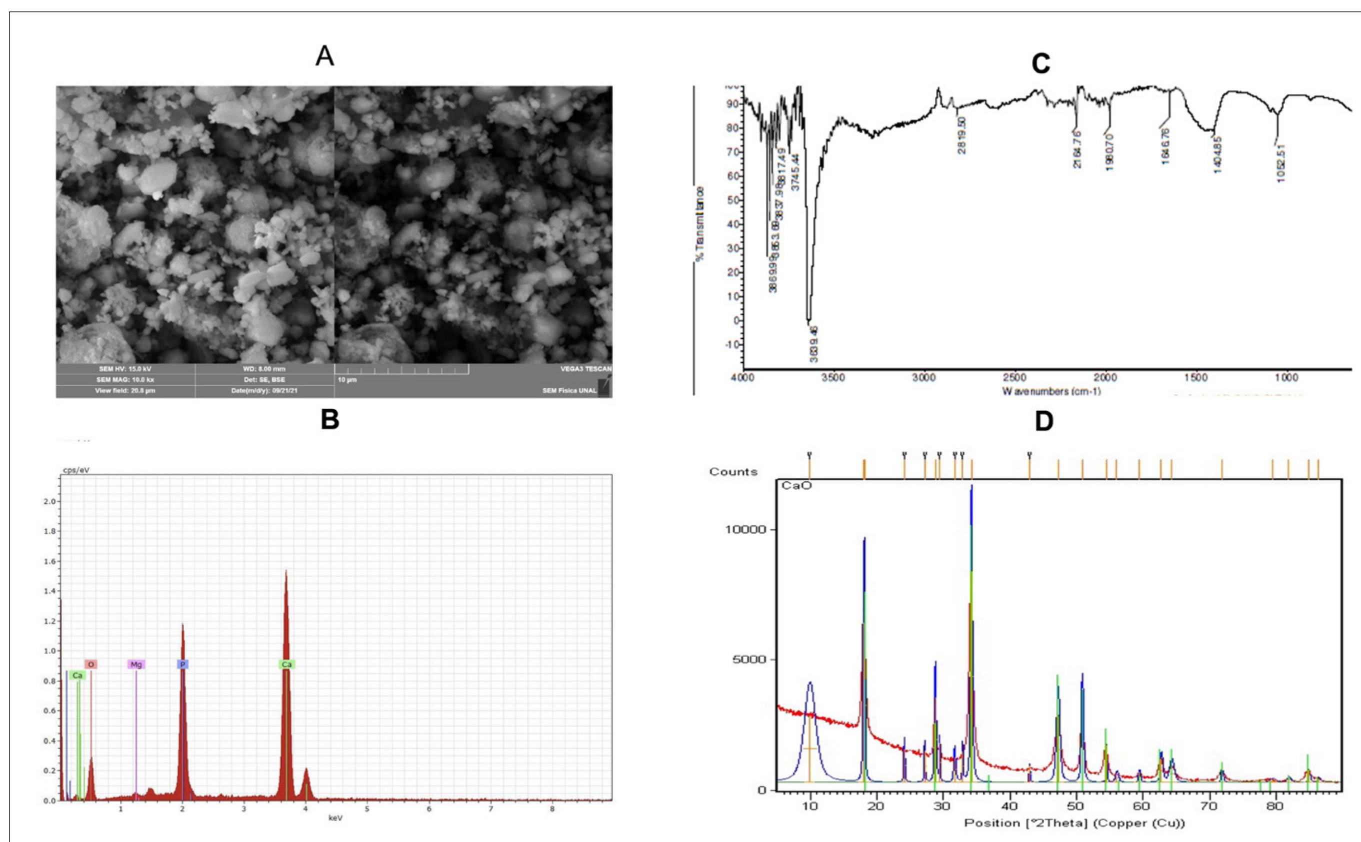

Figure 1
QCP5 Characterization: **(A** and **B)** SEM-EDS, **(C)** FTIR and **(D)** Diffractogram.

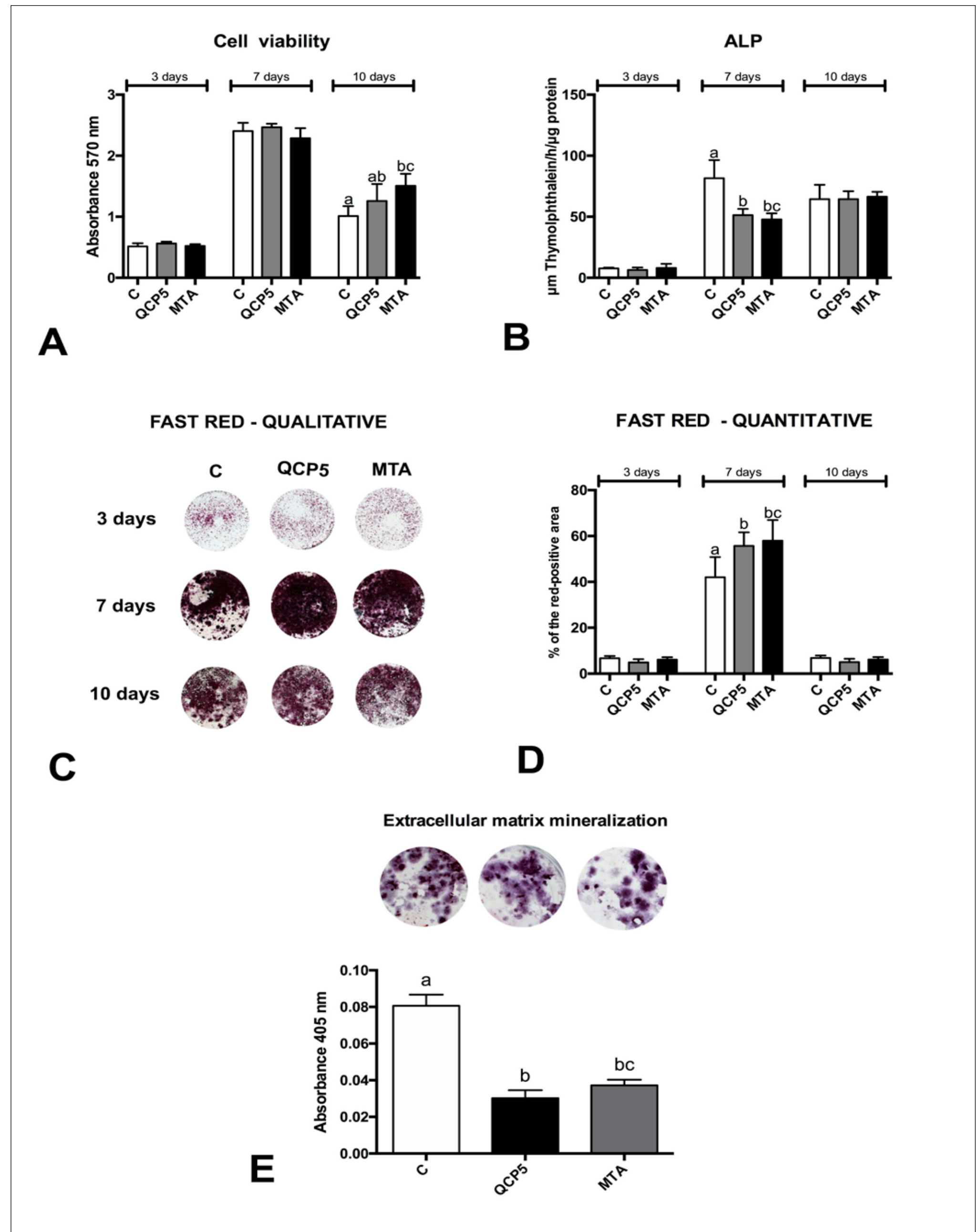

Figure 2
(A) Cell viability assessed by MTT assay at 3, 7, and 10 days of cell culture. **(B)** Activity of alkaline phosphatase through the release of thymolphthalein by hydrolysis of the thymolphthalein monophosphate substrate. **(C)** Qualitative detection of alkaline phosphatase by nuclear fast red *in situ* technique. **(D)** Quantitative detection of alkaline phosphatase by nuclear fast red *in situ* technique. **(E)** Qualitative and quantitative extracellular matrix mineralization. Control group, QCP5 (experimental ceramics), MTA (conventional material). Different letters represent statistical differences.

GraphPad Prism 6.0e software (Graph Pad software, Inc., San Diego, CA). The established significance level was 5%.

Results

Physicochemical characterization
Particles shown in the SEM-EDS figures are amorphous, round-edges, of multidisperse sizes composed of a submicrometric fraction but also a range of small particles aggregates of sizes up to 5 µm and large amorphous particles of around 5-10 µm figure 1A and 1B.
The following observations refer to figure number 1C containing the FTIR in which the band located at 3.642 cm^{-1} originates in the water OH stretch. The wide band at 3.433 cm^{-1} depicts adsorbed humidity water. At 1424 cm^{-1} the absorption is due to υ_3 the A-type substitution on carbonate anion. The 1.044 cm^{-1} strong absorption

peak is the bending mode of the PO_4^{-3} and the small shoulders are librations υ_1 and υ_3 of the same anion. The sharp weak absorption at 874 cm^{-1} comes from a calcium-deficient apatite (26), also from original phosphates anions substituted by carbonate B-type position after the research by Berzina-Cimdina (20) or from HPO_4^{-2} (27). All three sources seemingly different express the same structural finding. The peaks at 602 y 561 cm^{-1} arise from the υ_1 PO_4^{-1} libration or may also stem from the β-TCP following the findings reported by Elliot (26) fact to be taken into account since TCP is added to the QCP5. These findings are somewhat logical and compatible with the composition of the studied powder because the synthesis was conducted in air without excluding atmospheric CO_2 and the product was also kept in air and therefore there are B and A substitutions for carbonate. As indicated in the paper Berzina-Cimdina et al, these peak assignments are subjected to position and strength changes caused by synthesis route, chemicals employed, thermal treatments, treatment duration, particle size, crystallinity and final composition (20). This material coming from eggshell contains magnesiuem and therefore besides TCP it may contain some true whitlockite that may also exert an effect on the FTIR results.

In the diffractogram there are several medium to strong peaks originated in calcium hydroxide, it must be remembered that there is no direct relation between the counts per second or peak intensity and the concentration of two or more compounds since the instrumental sensitivity for each compound is different. Some other peaks such as those at 26, 32, 33, 47 and 49.5 belonging to different types of apatites. Some smaller peaks correspond to beta TCP and other phosphate phases (figure 1D).

Cell viability

At 3 and 7 days there was no statistically significant difference between the groups ($P=0.1585$, $P=0.1202$ respectively), while at 10 days there was a significant increase in cell viability in the MTA group in comparison to the control group ($P=0.0099$); however, there was no difference between the QCP5 group in comparison to the control group ($P=0.2180$) or MTA group ($P=0.2126$) (Figure 2A).

Analysis of ALP

At 3 days there was no significant difference between all three groups ($P=0.5337$). At 7 days the expression of ALP was higher in the control group in comparison to the other groups QCP5 ($P=0.0008$) and MTA ($P=0.0003$), however, no difference was observed between the latter two ($P=0.8324$). At 10 days there was no statistical difference between all three groups ($P=0.9102$) (Figure 2B).

In situ *ALP analysis by nuclear fast red*

Both qualitatively (Figure 2C) and quantitatively (Figure 2D) it was observed that at 3 days there was no significant difference between the groups ($P=0.5337$), and at 7 days the expression of ALP was significantly lower for the control group compared to the QCP5 ($P=0.0477$) and MTA ($P=0.0217$) groups, and between the latter two there was no difference ($P=0.8991$). At 10 days there was no statistical difference between all the groups ($P=0.0910$).

Detection and quantification of mineralized matrix

Figure 2E shows qualitatively and quantitatively that mineralized matrix deposits at 14 days of culture were similar between the QCP5 and MTA groups ($P=0.0845$). The control group showed a significantly elevated presence of mineralization nodules compared to the QCP5 and MTA groups ($P=0.0001$).

Immunofluorescence

Figure 3 shows the 3-day immunolocation of Alp, Col1a1, and DMP1 proteins in the control, QCP5, and MTA groups. Cell contour and/or cytoskeleton is evidenced by green coloration (phalloidine marking), blue nucleus (DAPI marking), and red protein presence (antibody marking). In the first row we can observe that the Alp protein was marked in a similar way for the three groups; it was present in the

cytoplasm and adjacent to the nucleus. The presence of Col1a1 is noticeably diminished in the MTA group; the opposite occurs in the cells of the control group and QCP5, which present a similar expression. However the immunolocation of DMP1 is more highlighted in the QCP5 group compared to the other groups.

Discussion

The cell viability results of this paper show that at 3 and 7 days there is no significant difference between the groups (control, QCP5, MTA) while at 10 days there was an increase in cell viability in the MTA and also the QCP5 groups in comparison to the control group; however, the QCP5 viability fell in between the control and the MTA groups showing non meaningful difference between the QCP5 group in comparison to the control group or MTA group. This result seems to indicate that MTA and QCP5 provide a good environment for cell survival, possibly due to bioavailability of mineral ions in concentrations larger than those of the control group. One of the most important proteins for the formation of a mineralized matrix is ALP, this must present a gradual increase in the initial phase of odontoblastic differentiation and indicates the potential for cell differentiation (28, 29). Therefore, the evaluation of this enzyme determines the bioactivity of the material and its potential to promote the onset of repair with the formation of mineralized tissue (8). The ALP biochemical method outcome shown in figure 2B displays a similar response at 3 days in all groups. At 7 days there is a net difference between the control group and the QCP5 and MTA groups. In a sense, the control group largely outperformed the two synthetic materials. A possible reason behind this odd behavior is probably once again the bioavailability of mineral ions. The ions in the culture broth are dissolved and readily available while the ion stock in both QCP5 and MTA are solid and must dissolve and diffuse a certain distance through the well bottom. This explanation is supported by the observation that at 10 days, the bioactivity of all three treatments is equivalent because the ion availability has overcome the solubilization and diffusion barriers. In figure 2C and 2D nucle-

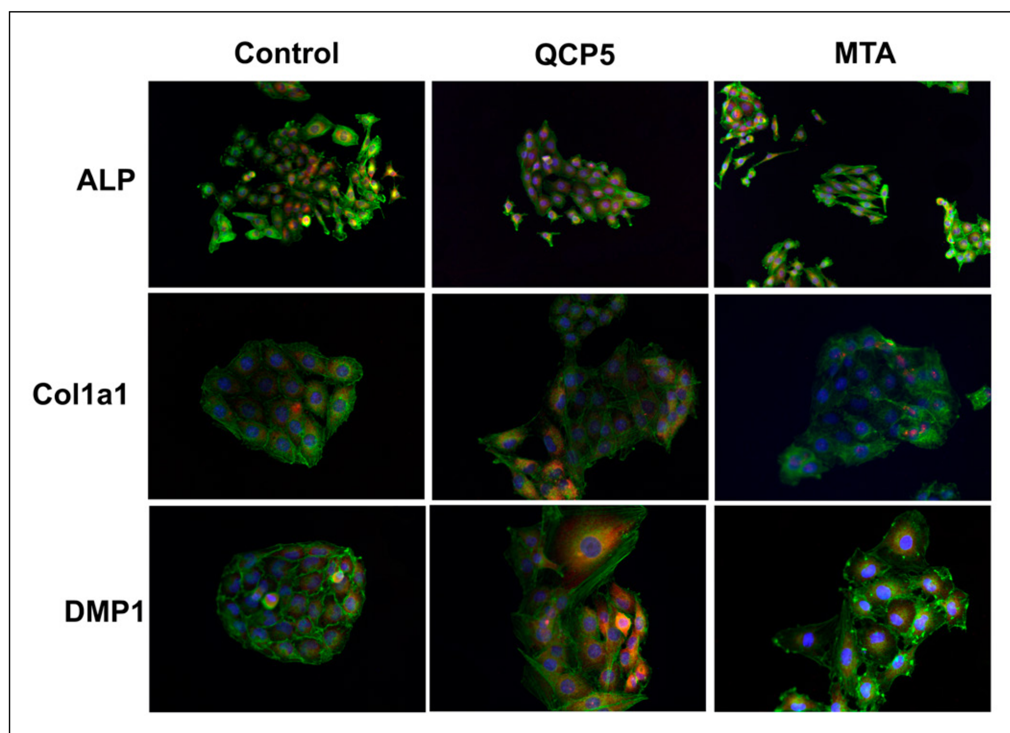

Figure 3
Immunolocation of Alp, Col1a1, and DMP1 proteins in OD-21 cells placed in indirect contact with QCP5 and MTA. Actin filaments marked in green (phalloidin), cell nucleus marked in blue (DAPI), and protein marked in red (antibody).

ar fast red quantitative results show that the three groups express differently at 7 days. At this time point, the QCP5 and MTA groups are clearly superior to the control group and QCP5 performs better than the MTA group as shown in figure 2C.
Experimental tests with alizarin red shows that the QCP5 promotes the formation of mineral nodules in a similar way to MTA; this implies that an intracellular deposition of calcium occurs and is subsequently released into the extracellular space. The control group showed a significantly elevated presence of mineralization nodules compared to the QCP5 and MTA groups probably because the cells in their natural state tend to mineralize.
The images obtained from the immunofluorescence assay showed similar expression of ALP in the three groups. The qualitative results at the three experimental times indicated that the MTA and QCP5 behave similarly. These results echo those found in a study with murine cells exposed to Biodentine, which found no significant differences between the groups treated with Biodentine and the control, favoring the odontoblastic pathway and the association with an intense secretory activity of the cell (17, 30).
The biological response depends on calcium which is the main component of ceramics and is related to bioactivity, osteoblastic survival, modulation of osteopontine levels, bone morphogenetic protein-2, and activation of ATP, which is important in the mineralization process (14, 31-33). Odontoblastic differentiation and the formation of mineralized tissues is regulated by the expression of proteins such as Col1a1 and Dmp1 (28) in the images obtained from our immunolocation observations, the presence of Col1a1 is notably diminished in the MTA group, and DMP1, is more marked in the QCP5 group compared to the other groups. This shows that proteins play a role in intracellular signaling which leads to the differentiation of mesenchymal cells as nucleating proteins in the extracellular matrix, and aids in the orientation and reinforcement of nanocrystals, which are essential properties for dentin regeneration (34).

Conclusions

Based on our results, we conclude that the bioceramic QCP5 promoted cell viability, the expression of ALP, Col1a1, and DMP1 and the formation of mineralization nodules. This indicates it is a promising material for dental pulp capping to conserve vital dental pulp tissue and, by extension, the natural tooth structure surrounding it.

Clinical Relevance

In office practice dental pulp injuries are treated with calcium hydroxide, calcium tri/disilicate and polymers. These materials differ largely from enamel composition (calcium phosphates). The ceramic QCP is mainly based on biomimetic calcium phosphates that closely emulate enamel electrolytes composition (Mg, chlorides, carbonates), it is biocompatible and has better physical and chemical properties. Its composition is like the human mineral component precursors. This paper presents some assays that demonstrate the QCP performance and usefulness.

Conflicts of Interest

The authors have no conflicts of interest to report.

Acknowledgements

Financial support was given by the Universidad Nacional de Colombia-Sede Bogotá and the São Paulo University-Brazil.

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