Manuscript Details

Manuscript number	DEMA_2017_806_R1
Title	Cellular differentiation, bioactive and mechanical properties of experimental light-curing pulp protection materials
Article type	Full Length Article

Abstract

Objectives: Materials for direct pulp capping/protection should have therapeutic properties in order to stimulate remineralization and pulp reparative processes. The aim of this study was to evaluate the mechanical properties, biocompatibility, cell differentiation and bioactivity of experimental light-curable resin-based materials containing bioactive micro-fillers. Methods: Four tailored calcium-phosphosilicate micro-fillers were prepared and incorporated into a resin blend: 1) Bioglass 45S5 (BAG); 2) polycarboxylate zinc-doped bioglass (BAG-Zn); 3) βTCP-modified calcium silicate (β-CS); 4) polycarboxylate zinc-doped β-CS (β-CS-Zn). These resins were tested for flexural strength (FS) and fracture toughness (FT) after 24h and 30-day storage in simulated body fluid (SBF). Cytotoxicity was evaluated using MTT assay, while bioactivity was evaluated using mineralization and gene expression assays (Runx-2 & ALP). Results: The lowest FS and FT at 24h was attained with β-CS resin, while all the other tested materials exhibited a decrease in FS after prolonged storage in SBF. β-CS-Zn maintained a stable FT after 30-day SBF aging. Incorporation of bioactive micro-fillers had no negative effect on the biocompatibility of the experimental materials tested in this study. The inclusion of zinc-doped fillers significantly increased the cellular remineralization potential and expression of the osteogenic genes Runx2 and ALP ($p<0.05$). Significance: The innovative materials tested in this study, in particular those containing β-CS-Zn and BAG-Zn may promote cell differentiation and mineralization. Thus, these materials may be suitable therapeutic pulp protection materials for minimally invasive and atraumatic restorative treatments.

Keywords	Bioactivity; Biocompatibility; Fracture toughness; Flexural strength; Cell differentiation; pulp protection.

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Dental Materials – Journal

Editor-in-Chief
Prof. Dr. David C. Watts

On behalf of my co-workers Ashvin Babbar, Borzo Gharibi, Victor Pinheiro Feitosa, Ricardo Marines Carvalho, Lidiany Karla Azevedo Rodrigues, Avijit Banerjee, Timothy Watson I have submitted our article with the title: **"*Cellular differentiation, bioactive and mechanical properties of innovative light-cured pulp protection restorative materials* "** to **Dental Materials**. This manuscript is original and it has not been published in any form or language, but it is only submitted for publication to **Dental Materials**. The authors are completely free of conflicts of interest.

Our groups of research collaborated to evaluate the mechanical properties, biocompatibility, cell differentiation and bioactivity of experimental light-curable resin-based materials containing bioactive micro-fillers after storage in simulated body fluid (SBF). The mechanical properties were assessed through the evaluation of the flexural strength (FS) and fracture toughness (FT). The cytotoxicity of the tested materials was tested using the MTT assay, while cell differentiation and mineralization were assessed using gene expression assays (Runx-2 and ALP). The bioactivity of the tested materials was evaluated using Raman spectroscopy and scanning electron microscopy (SEM)

We observed that the lowest FS and FT at 24h was attained with the most alkaline resin containing calcium-silicate fillers, while all the other tested materials containing bioglass and calcium silicate filler doped with zinc had a decrease in FS after prolonged storage in simulated body fluid (SBF). The resin-based materials containing bioglass and calcium-silicate fillers doped with zinc maintained a stable FT after 30-day SBF aging. Incorporation of bioactive micro-fillers had no negative effect on the biocompatibility of the experimental materials tested in this study. The inclusion of zinc-doped fillers significantly increased the cellular remineralization potential and expression of the osteogenic genes Runx2 and ALP ($p<0.05$).
Such innovative materials, in particular those containing the bioactive fillers doped with zinc, may induce cell differentiation and mineralization. Thus, these materials may be suitable therapeutic pulp protection materials for minimally invasive and atraumatic restorative treatments.

Thank you very much for your kind attention

Best Regards

Prof. Dr. Salvatore Sauro - Corresponding author

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AUTHOR DECLARATION – CONFLICT OF INTEREST AND INDIVIDUAL CONTRIBUTION

All authors have contributed significantly, and they are all in agreement with the manuscript. They gave full permission to publish photographs in all forms and media

We declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

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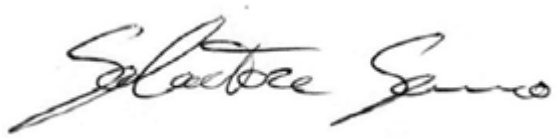
Prof. Dr. Salvatore Sauro



Dr. Ashvin Babbar

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Prof. Dr. Victor Pinheiro Feitosa

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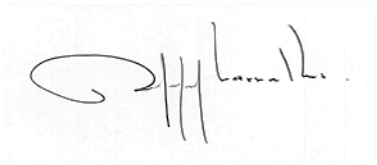
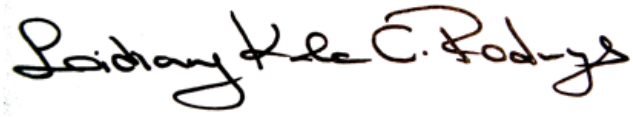
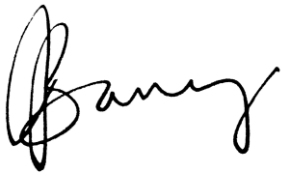


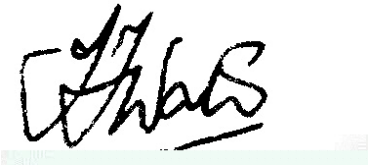
Prof. Dr. Lidiany Karla Azevedo Rodrigues



Prof. Dr. Avijit Banerjee



Prof. Dr. Timothy F Watson



MANUSCRIPT NUMBER: DEMA - 2017 - 806

Dear Editor and Reviewers,

Herewith our answers to the comments on the manuscript titled: “Cellular differentiation, bioactive and mechanical properties of innovative light-cured pulp protection restorative materials”.

A revised version of this manuscript has been also enclosed considering all the suggestions kindly offered by; all changes have been highlighted throughout the entire document.

In this regard, we would like to show to the Editor and Reviewers all our gratefulness for their constructive comments that were fair and helpful.

In this interesting paper the authors assessed the mechanical properties, biocompatibility, cell differentiation and bioactivity of experimental light-curable resin-based materials containing bioactive micro-fillers. However, after reading this manuscript, several concerns and doubts have arisen. Therefore, this Reviewer has specific criticisms on the manuscript and would like to ask the authors to pay attention to the following comments, concerns and suggestions about this paper.

1. A number of spelling mistakes was observed along the text of this manuscript: (dentin x dentine) (... and formation of dentine bridge formation with ...) etc. Please, revise the whole manuscript.
   - Our native English-speakers have revised the whole manuscript and corrected all the grammar and spelling mistakes.

2. It is known and very well established that resin-based dental products are not indicated for direct application on pulp exposures. Therefore, I request the authors to state clearly in the Introduction and Discussion Sections of this paper that the experimental light-curable resin-based materials with bioactive micro-fillers assessed in this study have not been developed to be used as pulp-capping agents.
   - It is important to consider that nowadays new resin-based materials have been formulated for pulpal-capping purposes, such as resin-modified Portland cement–based materials such as Theracal LC, (BISCO, Chicago, IL, USA), a light-curable material advocated for direct and indirect pulp capping, although this resin-modified Portland cement–based material was demonstrated to induce reduction in cell metabolism and protein expression and greater cell toxicity compared to conventional calcium silicate cements (see references 14 and 15 in our paper). However, since we approve the concern underlined by this referee, we have introduced the following statement in the discussion: “ Pulp-protection m*aterials used in the atraumatic restorative treatment (ART) technique should be able to evoke remineralization with no inflammatory pulp response [9-13]. Accordingly, this study aimed at generating advanced light-curable resin-based materials containing different types of experimental bioactive micro-fillers: evaluating their flexural strength, fracture toughness, biocompatibility, cell differentiation and bioactivity after storage in simulated body fluid (SBF). However, it is important to anticipate that the experimental light-curable resin-based materials with*

bioactive micro-fillers assessed in this study have not been developed to be used as direct pulp-capping agents."

3. Resin-modified glass-ionomer cements (RMGICs), such as Vitrebond (3M ESPE), have been widely recommended as liners, even for very deep cavities. Why the authors did not use a RMGIC as a control group ?

- The observation of this reviewer is correct, however we did not want to involve any commercial material in our study. We actually aimed at demonstrating how the incorporation of specific bioactive fillers in resin-based materials could influence the mechanical properties, biocompatibility, cell differentiation and bioactivity of experimental pulp-protection materials after storage in simulated body fluid (SBF). Moreover, since we used a direct cell-seeding method for our biocompatibility and cell differentiation tests, it could not represent the most suitable method for GIC to achieve at the end of the study "appropriate results", in terms of cell survival. It is also well-known that GIC-based material should not be applied in case of indirect pulpal capping unless after application of a protective layer of calcium hydroxide.

  REFERENCES supporting our observation:

  Cytotoxicity of modified glass ionomer cement on odontoblast cells. Chen S, Mestres G, Lan W, Xia W, Engqvist H. J Mater Sci Mater Med. 2016 Jul;27(7):116. doi: 10.1007/s10856-016-5729-y.

  Cytotoxicity of resin-based luting cements to pulp cells. Pontes EC, Soares DG, Hebling J, Costa CA. Am J Dent. 2014 Oct;27(5):237-44.

4. In my opinion and based on scientific data, the dental materials selected to be applied on caries-affected dentin (after mechanical caries-infected removal) should present antibacterial property. Then, I would like to know why the authors did not evaluate the antibacterial activity of these experimental resin-based products ?

- The observation of the reviewer is really appropriate. Indeed, the reason why we did not included any result about antibacterial effects of such experimental materials is that we have an ongoing project about such a specific topic. We have already stated with the evaluation of the remineralizing properties of our experimental materials in real caries-affected dentine in extracted teeth prepared with a chemio-mechanical method (Carisolv), as well as in simulated bacteria-mediated caries affected dentine prepared in sound extracted human teeth. In this further study we are about to perform some tests to evaluate if our experimental materials are able to induce any bacteriostatic of bactericidal effect on different cariogenic biofilms. Thank you so much for highlight the importance to perform antibacterial tests in such a specific scenario.

5. Why did the authors polish the resin-disk specimens with SiC papers up to #1000-grit under continuous distilled water (DW) irrigation before placing them in wells of 24-well plates? May this procedure remove the highly toxic uncured monomers from the specimens' surface, interfering with the results of the biological test employed in this in vitro study? This topic should be included in the Discussion Section.
   - We are afraid we have to disagree with this observation. The uncured and highly toxic monomers are usually present on the outer surface of the resin due to inhibition effect of oxygen during light-curing procedures. Conversely, when these materials are usually applied in thin layers (~ 1 mm) onto the floor of the cavity and in contact with the residual dentine, such an inhibition effect does not occur in any side of the material. The only case to attain an oxygen-inhibition effect is at the end of the restoration if no oxygen-guard agent (e.g. glycerin) is applied during the final light-curing step. Moreover, as far as we know, in most of the studies published in literature, the specimens are always polished before testing.

6. The authors also reported that the specimens were decontaminated by soaking them in absolute ethanol for 10 min. Again, this procedure may remove toxic compounds from resin-based materials. I recommend the authors to use another decontamination protocol in further studies.
   - Unfortunately, we have to be repetitive in our answer as it is the same gave for the query number 5. Actually, soaking the specimens in ethanol may sometime makes the specimens more cytotoxic as per its effect to induce elution of unreacted monomers and decrosslinking effect; however, this latter situation does not really occur within 10 minute of immersion in ethanol, but it usually takes more than 12 h to induce substantial changes. However, we would like to thank this referee for his recommendation, and indeed we will use an alternative decontamination protocol in further studies.

7. Why the authors did not use human dental pulp stem cells (DPSCs) in this in vitro study ? Did the authors confirm the phenotype of the mesenchymal stem cells (MSCs, Lonza Biologics, Slough, Berkshire, UK) before conducting this investigation?
   - The reason why we used MSCs from Lonza Biologics was because ethics approval was not required for use of commercial cells. Indeed, during the period of our experiments in our department there were several difficulties regarding the collection and the use of hDPSCs. However, in this situation is solved nowadays and in our future studies we will be able to use hDPSCs. Regarding the phenotype and differentiation potential of the cells used in this study was already described in previous publications: "*Stem Cell Res Ther. 2017; 8: 103. Published online 2017 Apr 27. doi: 10.1186/s13287-017-0552-z. Human mesenchymal stem cells maintain their phenotype, multipotentiality, and genetic stability when cultured using a defined xeno-free human plasma fraction*"
     Indeed, these MS-cells presented the normal hMSC phenotype, being negative for CD14, CD19 and positive for CD29, CD44, CD73, CD90, CD105, CD166 and Stro-1
     This information was included in the materials&methods session and the reference added in the references list.

8. Table 2 is related to MTT assay and mRNA gene expression data. However, it was described as – "Table 2. Flexural Strength (FS) and Fracture Toughness (FT) of experimental resins after 24 h and 30 d ageing in AS at 37°C."
   - Table 2 has been revised and corrected as per indication of the reviewer.

9. In order to facilitate the readers, the events described in the Legends of the Figure 1 (MEV – I and L) and Figure 2 (fluorescence – A,B,C, and D) should be pointed in the images.
   - Pointers and arrows have been introduced in images and caption as wisely suggested by the reviewer.

10. In this in vitro study, the authors described that "The aim of this study was to evaluate the mechanical properties, biocompatibility, cell differentiation, and bioactivity of experimental light-curable resin-based materials containing bioactive micro-fillers". Therefore, the authors cannot conclude which materials are adequate or possible alternatives for clinical application. (Please, see the Conclusion Section – "In terms of biocompatibility and osteoinductivity/dentinogenesis, the resins confining the zinc-doped fillers (β-CS-Zn and BAG-Zn) were the most promising as possible alternative pulp protection materials for use clinically."

    - The sentence highlighted by the this reviewer in the conclusions session has been modified as following "

11. The references are not standardized. Only a few examples:

25. Ilie N, Hilton TJ, Heintze SD, Hickel R, Watts DC, Silikas N, et al. Academy of Dental Materials guidance-Resin composites: Part I-Mechanical properties. Dent Mater 2017;33:880-894.

26. Salgado VE, Cavalcante LM, Moraes RR, Davis HB, Ferracane JL, Schneider LF. Degradation of optical and surface properties of resin-based composites with distinct nanoparticle sizes but equivalent surface area. J Dent 2017;59:48-53.

27. Ferracane JL. Hygroscopic and hydrolytic effects in dental polymer networks. Dent Mater 2006; 22: 211-22

28. Shahwan T, Zünbül B, Tunusoğlu O, Eroğlu AE. AAS, XRPD, SEM/EDS, and FTIR characterization of Zn2+ retention by calcite, calcite–kaolinite, and calcite–clinoptilolite minerals. J. Colloid Interface Sci 2005; 286: 471–478.

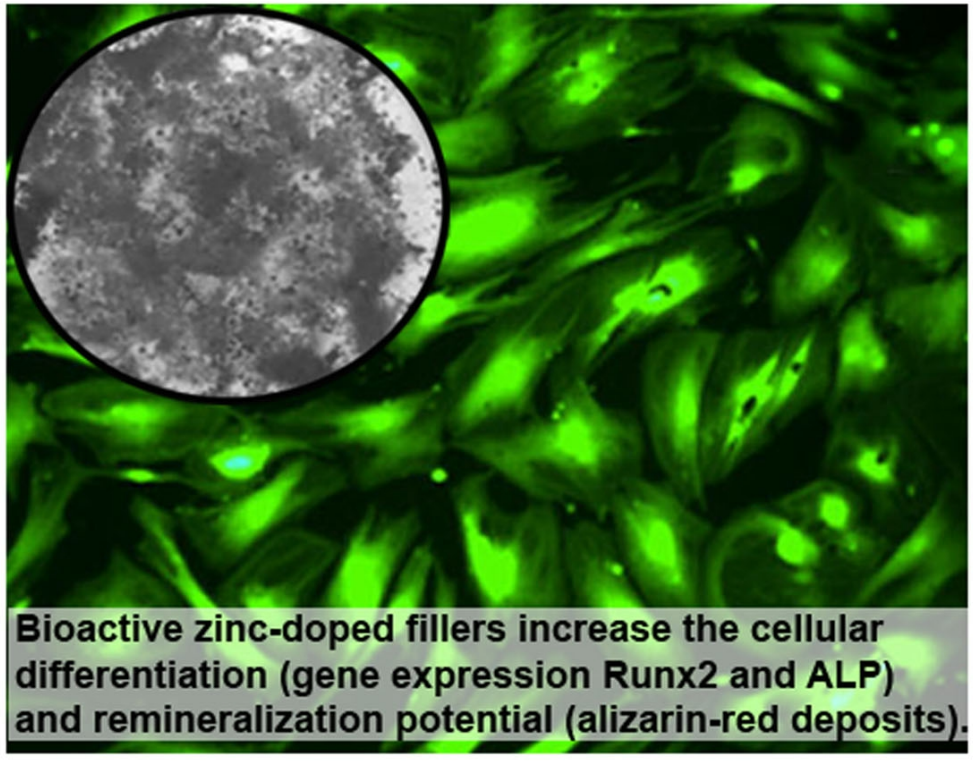
- We have now revised and standardized all the references.

We hope these changes have made the manuscript acceptable for publication. We thank the reviewers for helping us to improve the paper.

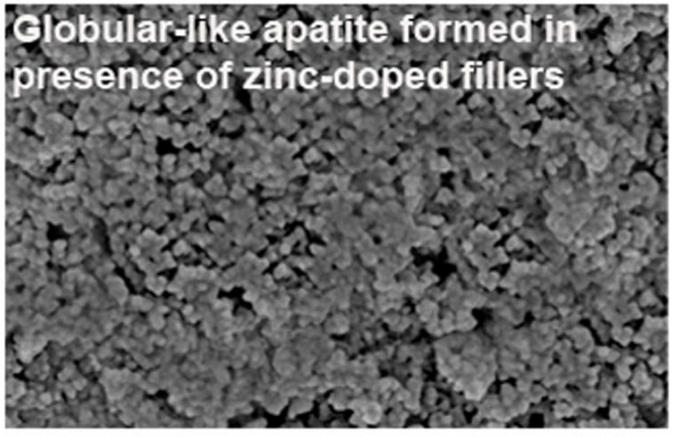
Best Regards

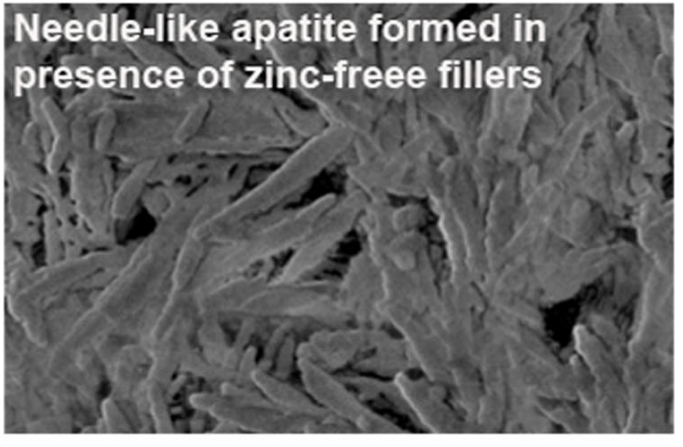
HIGHLIGHTS

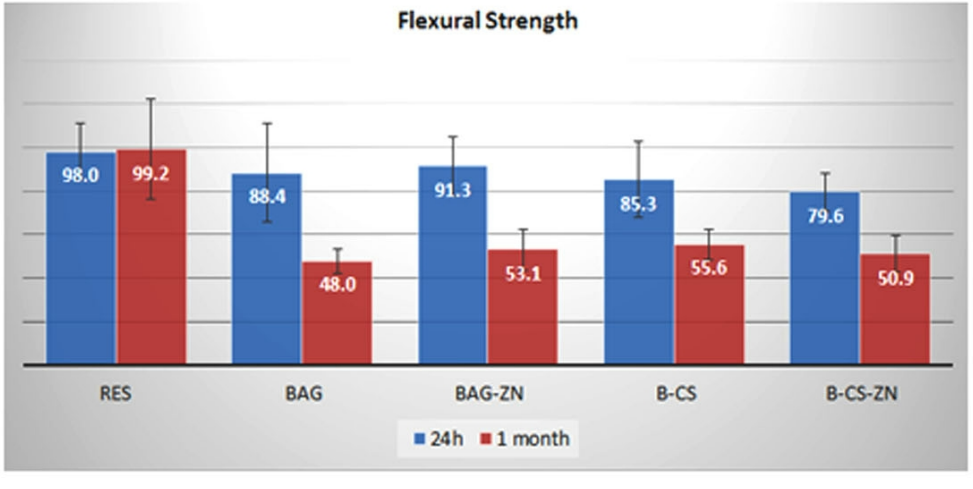
- Incorporation of bioactive fillers into resin-based materials induces apatite precipitation
- Resins containing zinc-doped bioactive fillers maintain stable mechanical properties over time.
- Zinc-doped bioactive fillers have excellent osteo-inductivity/dentinogenesis and biocompatibility properties



Bioactive zinc-doped fillers increase the cellular differentiation (gene expression Runx2 and ALP) and remineralization potential (alizarin-red deposits).







Resins containing zinc-doped bioactive fillers maintain stable mechanical poperties over time compared to those resins with zinc-free bioactive fillers

ACKNOWLEDGEMENTS

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Cellular differentiation, bioactive and mechanical properties of experimental light-curing pulp protection materials

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ABSTRACT

Objectives: Materials for direct pulp capping/protection should have therapeutic properties in order to stimulate remineralization and pulp reparative processes. The aim of this study was to evaluate the mechanical properties, biocompatibility, cell differentiation and bioactivity of experimental light-curable resin-based materials containing bioactive micro-fillers.

Methods: Four tailored calcium-phosphosilicate micro-fillers were prepared and incorporated into a resin blend: 1) Bioglass 45S5 (BAG); 2) polycarboxylate zinc-doped bioglass (BAG-Zn); 3) βTCP-modified calcium silicate (β-CS); 4) polycarboxylate zinc-doped β-CS (β-CS-Zn). These resins were tested for flexural strength (FS) and fracture toughness (FT) after 24h and 30-day storage in simulated body fluid (SBF). Cytotoxicity was evaluated using MTT assay, while bioactivity was evaluated using mineralization and gene expression assays (Runx-2 & ALP).

Results: The lowest FS and FT at 24h was attained with β-CS resin, while all the other tested materials exhibited a decrease in FS after prolonged storage in SBF. β-CS-Zn maintained a stable FT after 30-day SBF aging. Incorporation of bioactive micro-fillers had no negative effect on the biocompatibility of the experimental materials tested in this study. The inclusion of zinc-doped fillers significantly increased the cellular remineralization potential and expression of the osteogenic genes Runx2 and ALP ($p<0.05$).

Significance: The innovative materials tested in this study, in particular those containing β-CS-Zn and BAG-Zn may promote cell differentiation and mineralization. Thus, these materials may be suitable therapeutic pulp protection materials for minimally invasive and atraumatic restorative treatments.

Key words: Bioactivity; Biocompatibility; Fracture toughness; Flexural strength; Cell differentiation; pulp protection.

1. INTRODUCTION

The operative treatment of deep carious lesions can be challenging especially when approaching the pulp as the increased risk of pulp exposure during excavation can reduce the probability of pulp survival [1]. An ideal pulp protection material for such scenarios should be highly biocompatible and bioactive [2, 3]. This is especially relevant with the contemporary minimally invasive restorative philosophy where contaminated (caries-infected) dentin is removed from deep cavity selectively, retaining most of the demineralized but repairable (caries-affected) dentin for potential remineralization as well as avoiding pulp exposure [4, 5]. Indeed, clinicians are increasingly relying on restorative ion-releasing materials such calcium silicate cements to seal the restored interfaces in order to help remineralize the caries-affected tissues [6].

In certain clinical cases, indirect and/or direct pulp protection may help maintain pulp sensibility by facilitating healing/repair. Materials used for this purpose should interact with the pulp cells to stimulate the formation of reparative dentin [7-9]. Calcium silicate and MTA-like cements have been used as they can encourage remineralization and dentin bridge formation with no/minimal inflammatory pulp response [9, 10, 11]. However, their use as a bio-interactive restorative material is limited due to shortcomings in their mechanical properties, setting time and dissolution rate [12, 13]. It is hypothesized that the formulation of resin-modified bioactive cements might present a solution to combine the best of both resin-based technology with the bioactivity of such cements. Indeed, resin-modified Portland cement–based materials such as Theracal LC (BISCO, Chicago, IL, USA), a light-curable material advocated for direct and indirect pulp protection, showed greater compressive and flexural strengths compared to conventional calcium silicate cements, being more able to resist fracture during immediate placement of a definitive overlying restoration [14]. Nevertheless,

such a resin-modified Portland cement–based material demonstrated a reduction in cellular metabolism and protein expression. It was also exhibited greater cytotoxicity compared to conventional calcium silicate cements (15).

Sodium calcium phosphosilicates (e.g. Bioglass 45S5, BAG) have been used successfully in orthopedics as regenerative materials for bone [8, 16]. In particular, BAG has been shown to induce calcium-phosphate precipitation, subsequently converting to hydroxyapatite-like crystallites [8, 17]. Within dentistry, BAG is used in toothpastes and also as powders for dental air-abrasion/polishing to remineralize the dental hard tissues as well as treating dentin hypersensitivity [18, 19]. Bioactive glasses are so called due to their *in loco* remineralization of tissues, but they cannot be used in as a definitive dental restorative material unless incorporated into a resin-based matrix [20, 21].

The aim of this study was to evaluate the mechanical properties, biocompatibility, cell differentiation and bioactivity of experimental light-curable resin-based materials containing bioactive micro-fillers for their potential use as indirect pulp protection materials, after storage in simulated body fluid (SBF). The mechanical properties were assessed through the evaluation of their flexural strength (FS) and fracture toughness (FT). The cytotoxicity of the tested materials was tested using MTT assay, while cell differentiation and mineralization were assessed using gene expression assays (Runx-2 and ALP). The bioactivity of the tested materials was evaluated using Raman spectroscopy and scanning electron microscopy (SEM). The null hypotheses tested in this study were that the addition of bioactive micro-fillers: 1) would have no effect on mechanical properties of the experimental resin-based materials tested; 2) would neither increase cytotoxicity nor induce differentiation in primary human mesenchymal stem cells (MSCs).

2. MATERIALS & METHODS

2.1 Formulation of the resin-based bioactive materials

A control filler-free resin (RES) was made using three hydrophobic monomers (55 wt% urethane dimethacrylate and, 4.5 wt% bisphenol A diglycidildimethacrylate, 10.5 wt% triethylene glycol dimethacrylate, Sigma-Aldrich, Gillingham, UK), 20 wt% 2-Hydroxyethyl methacrylate (Sigma-Aldrich), 10 vol% absolute ethanol (Sigma-Aldrich) and a photo-initiating complex comprising 0.5wt% camphorquinone / 1.0 wt% ethyl 4-dimethylaminobenzoate (Sigma-Aldrich). The experimental ion-releasing resins were formulated using 40 vol% micro-filler and 60 vol% resin blend [21]. Four experimental light-curable resin-based materials containing tailored bioactive micro-fillers were formulated as described by Sauro *et al*. [21]. In brief, Bioglass 45S5 (BAG) micro-filler (<20 µm size) was sintered and incorporated within the composition of the control light-curable resin blend. The second micro-filler, BAG-Zn (<20 µm), was created by modifying the composition of the BAG with 20 wt% zinc oxide (ZnO: Sigma-Aldrich) and a 10 wt% polycarboxylic acid solution (PAA: M_w 1800; Sigma-Aldrich) and finally incorporated into the light-curable resin blend. The third micro-filler (β-CS) was formulated by modifying the composition of a type I ordinary Portland cement (OPC: Italcementi Group, Cesena, Italy) by adding 10 wt% β-tri-calcium phosphate [βTCP: $Ca_3(PO_4)_2$ (Sigma-Aldrich)]. The cement was mixed in deionized water (ratio 2:1 powder/liquid) and allowed to set in an incubator at 37°C for 24h. The set cement was subsequently ground and sieved as described previously [30] until <20 µm particle size was achieved. The fourth micro-filler (β-CS-Zn) used in this study was created by mixing 80 wt% OPC, 20 wt% ZnO and 10 wt% βTCP in H_2O (ratio 2:1). After setting for 24 h at 37°C, the micro-filler was treated (ratio 1:1) in a polymer flask with a 10 vol% polyacrylic acid (PAA) solution for 15 min (45 rpm) and incubated in a furnace at 40°C for 12 h. The resulting product was finally re-ground and sieved

(<20 µm). These latter micro-fillers were incorporated also into the light-curable resin blend as previously described.

2.2 Flexural strength test (FS)

Light curing resin-based specimens prepared in accordance with ISO 4049 (25 mm x 2 mm x 2 mm) may receive a non-uniform distribution of energy during the photo-polymerization procedure, which may in turn, affect their flexural strength. Therefore, smaller specimen (10 mm × 2 mm × 2 mm) were used in this study to reduce the variable effect of distance on resin polymerization as described in a previous study [22]. The experimental resin-based materials were inserted into rectangular silicone molds. A glass slide along with a plastic strip were placed on top to achieve parallel surfaces with no air bubbles. The specimens were light-cured (>1000 mW/cm^2) from both sides for 1 min using a LED light curing unit (Litex 695, Dentamerica Inc., Industry, CA, USA). Twelve specimens (n=12) were prepared for each experimental resin cement as well as the control resin blend. The specimens were stored for 24h at room temperature, half (n=6) were tested, and the other half (n=6) were further incubated for 30 d at 37°C in simulated body fluid solution (SBF) [21]. All samples were tested using a universal testing machine (Instron 5569) with a 500 N load cell and crosshead speed of 0.5 mm/min using a 3-point bending system. The maximum fracture load (Newton) for each specimen was recorded and the flexural strength in MPa was calculated according to the equation:

Flexure strength = $3FL/2bd^2$, where W=width and H=height of the specimens.

Two-way ANOVA statistical analysis with a significance level of 0.05 and a Tukey's post-hoc test were performed with the resin blends as independent variables and the FS as the dependent variable (Sigma Stat® , Version 3.5, Systat Software Inc., Point Richmond, USA).

2.3 Fracture toughness test (FT)

The FT (K_{IC}) was measured according to the ASTM standards (E 399-83), using single-edge notched-beam specimens [23]. Twelve specimens (16 mm × 2 mm × 2 mm) for each experimental group were created using bar-shaped silicone molds and by compressing the material using a glass slide and finally light-cured as described previously. After removal from the mold, the specimens were ground with silicon carbide sand paper (grit size # 1200/4000) to remove any excess material on the specimens' edges. All specimens were then stored in distilled water at 37°C prior to testing for 24 h. A notch (0.3 mm wide, 1 mm deep) was created for each specimen with a diamond saw under water irrigation. The width of the notch was determined by the thickness of the blade, whereas the standardization of the notch's depth was assured by a sliding depth-gauge mechanism installed on the saw, to 1 mm intrusion. The depth of the notch was measured with a digital microscope (CY-800B, Tokyo, Japan). The resin specimens were subjected to the same two environments, 24 h (n=6) at room temperature and incubated for 30 d (n=6) at 37°C in SBF. Specimens were tested using an Instron 5569 machine in a 3-point bending test, where the supporting length [(*S*)=4W] was 16 mm. The samples were loaded until failure, using a crosshead speed of 0.5 mm/min. During testing, the specimens were immersed in distilled water at room temperature. The force during bending of the specimen was measured as a function of its deflection. Load versus deflection plots were recorded and the maximum load (*P*) before failure was measured. The height (B) and width (W) of the specimens were measured with a micrometer and the notch depth (a) with a measuring microscope. The K_{IC} was calculated according to the equation shown below, from measurements with the single-edge notched-beam specimens (MPa.m^0.5).

$$K_{IC} = \frac{3\sqrt{a/W}\left[1.99 - a/W(1 - a/W)\left(2.15 - \frac{3.93a}{W} + 2.7(a/W)^2\right)\right]PS}{2(1 + 2a/W)\sqrt{(1 - a/W)^3 BW^3}}$$

Two-way ANOVA statistical analysis with a significance level of 0.05 and a Tukey's post-hoc test were performed with the resin blends as independent variables and the FS as the dependent variable (Sigma Stat® , USA).

2.4 Bioactivity evaluation through Raman spectroscopy and SEM

The specimens used in the FS test were also analyzed at 24 h and after 30 d of SBF storage using Raman spectroscopy and SEM. In brief, three specimens for each group were scanned in wet conditions using a computer-controlled confocal laser Raman spectro-microscope (Horiba Scientific Xplora, Villeneuved'Ascq, France) equipped with optical X20 objective and CCD detector attached to a modular research spectrograph. A near-infrared diode laser spot-size of ≤1 γm operating at 785 nm was used to induce the Raman scattering effect. Raman signals were acquired using a 600-lines/mm grating centered between 500-1000 cm^{-1}. The calibration of wavelength and intensity was performed according to manufacturer's specification using a silicon standard and the calibration system integrated with the software [17, 24]. One surface of each specimen was scanned in three different areas (ROI: 1 mm x 1 mm), and then submitted to K-means cluster (KMC) analysis as described by Sauro *et al* [5], using multivariate analysis, which included statistical patterning to derive independent clusters. The biochemical content of each cluster was analyzed using the average cluster spectra. Principal component analysis (PCA) data were set into a bilinear model of linear independent variables, the so-called principal components (PCS). Two to four clusters were identified but only two representative clusters such as resin and mineralization (peak at 961

cm^{-1} as reference) were obtained independently and considered in this study. All the specimens used for Raman spectroscopy analysis were dehydrated in increasing concentrations of ethanol (50% – 20 min; 75% – 20 min; 90% – 30 min; 95% twice – 30 min; 100% twice – 30 min) and desiccated for 24 h at 3°C. They were mounted on aluminum stubs using carbon tape, gold sputter coated and finally analyzed through FEG-SEM (S4000 Hitachi, Tokyo, Japan) at 3 kV.

2.5 Evaluation of the alkalinizing activity (pH)

Three resin-disk specimens were prepared for each tested material using silicon molds (Ø= 6 mm; h= 1 mm) and light-cured for 40 s using a LED curing system (Litex 695, USA) as previously described (see Section 2.4). The specimens were immersed in 25 ml of deionized H_2O (pH 6.8) in polypropylene-sealed containers stored at 37 °C. The pH/alkalinizing activity was evaluated using a professional pH electrode (Mettler-Toledo, Leicester, UK) at 37°C after 1 d, 15 d, 30 d. The H_2O was replaced over each measurement period.

2.6 Cell culture

Human mesenchymal stem cells basal medium (MSCs: ref. PT3238, Lonza), (Lonza Group Ltd, Basel, Switzerland) [25] were cultured in α-Minimal Essential Medium (MEM) containing penicillin (50 U/ml), streptomycin (50 µg/ml) (all from Sigma–Aldrich, Poole, Dorset, UK), Glutamax (2 mM) (Invitrogen, Paisley, UK) and 10% fetal bovine serum (FBS) (Sigma–Aldrich); cells were maintained at 37°C in a humidified 5% CO_2:95% air atmosphere. Three resin-disk specimens were prepared for each tested material using silicon molds (Ø= 6 mm; h= 1 mm) and light-cured for 40 s using a LED curing system (Litex 695, Dentamerica Inc., Industry, CA, USA). Subsequently, the specimens were polished using SiC papers up to #1000-grit under

continuous distilled water (DW) irrigation. The specimens were then decontaminated by soaking in absolute ethanol for 10 min. Cell monolayers were seeded into 24-well plates with $5x10^4$ cells/ml and cultured in the presence of the experimental materials for 14 days immersed in an osteogenic media supplemented with 0.1 µM dexamethasone, 0.05 mM Ascorbic Acid (AA) and 10 mM glycerophosphate (Sigma–Aldrich).

2.7 Biological testing (MTT) and osteogenic differentiation (Runx-2 and ALP)

Assessment of the biocompatibility of the experimental bioactive resin-based materials was performed in accordance with the ISO 10993-5 using a direct cytotoxicity test, MTT assay. In brief, after 14 days the media was replaced with a MTT solution (Methylthiazolyldiphenyl-tetrazolium bromide; Sigma-Aldrich) and incubated for 4 h at 37°C. Subsequently, the MTT solution was replaced with DMSO (Dimethyl sulfoxide, 99.9%, Sigma-Aldrich), and kept under continuous agitation (5 min) using an automatic chemical shaker (Titertek, Flow Laboratories). Finally, the absorbance of the MTT was measured on a microplate reader (Opsys MR, Dynex) at 570 nm wavelength with reference wavelength at 630 nm. One-way ANOVA and Tukey's test ($p<0.05$) were performed to analyze the data (Sigma Stat®, USA). Live/Dead fluorescent staining was performed to assess visually the effect of the resin blends on the MSCs. Here, solutions of 2 uM calcein AM (Sigma-Aldrich) and 4 µM ethidium homodimer in Dulbeccos's Phosphate Buffered Saline (PBS, Sigma-Aldrich) were used, the system then incubated for 10 min and assessed visually qualitatively using an Olympus X51 microscope coupled with an Olympus V-RFL-T camera, 10x magnification.

To assess osteogenic differentiation, mRNA expression of the markers of differentiation (i.e. *Runx-2* and *ALP*) was determined by quantitative real-time polymerase chain reaction (RT-qPCR), while accumulation of calcium deposits was visualized by staining using Alizarin Red.

Briefly, cells were fixed (15 min with 4% formaldehyde in PBS), stained for 10 min with alizarin red S (1:100 dilution in H_2O) and washed in 50% ethanol and air-dried.

Total RNA was extracted using TRI reagent (Ambion, Warrington, UK) and Phase Lock Gel Heavy tubes (Five-prime VWR, Leicestershire, UK) according to the manufacturer's instructions. After RNA purity and quantity was assessed by nanodrop (Fisher Scientific, London, UK) (A_{260}/A_{280} 1.8-2 was considered suitable for further analysis), possible contaminating DNA was removed and cDNA prepared from 1 µg RNA using QuantiTect Reverse Transcription Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. RT-qPCR was performed on a Mx3000P real time PCR system using Brilliant III Ultra-Fast SYBR Green qPCR Master mix (Stratagene, Agilent Technologies, Cheshire, UK) and the following primer pairs (5` to 3`) Runx-2 (AATGGTTAATCTCCGCAGGTC and TTCAGATAGAACTTGTACCCTCTGTT); ALP (AACACCACCCAGGGGAAC and TGGCATGGTTCACTCTCGT). PCR conditions consisted of 1 cycle of 95°C for 3 min and 40 cycles of 95°C for 10 s and 60°C for 20 s. RPL13a was used as an invariant housekeeping gene. One-way ANOVA and Tukey's test ($p<0.05$) were used to analyze the data (Sigma Stat®, USA).

Alizarin red staining (ARS) staining was performed to determine the mineralization ability of the tested materials on the cells in each well plate (n=3). After 14 days of incubation, the cells were rinsed, fixed with 10% formaldehyde for 30 minutes, and stained with 40mM alizarin red S (pH 4.2) for 30 minutes. After staining, the morphology was observed using light microscopy (Olympus lX71, Shinjuku, Tokyo, Japan).

3. RESULTS

3.1 Flexural Strength (FS) and Fracture Toughness (FT)

The mean and standard deviation of the results obtained with the flexural strength (MPa) and fracture toughness (MPa.m^0.5) are depicted in Table 1. At 24 h there was no significant difference in FS between the experimental resins and the control filler-free resin (RES), ($p>0.05$). However, all the tested experimental resins showed a significant drop in FS after AS storage for 30 d ($p<0.05$); there was no significant FS reduction in the RES specimens ($p>0.05$). Mean FS was affected by the material type ($F = 99.61$; $P < 0.001$), and by storage time ($F = 56.9$; $P < 0.001$).

At 24 h there was no significant difference in FT between the experimental resins and the RES group ($p>0.05$). Conversely, only the RES, experimental resin-based materials BAG and β-CS showed a significant drop in FT after prolonged AS storage ($p<0.05$). The other two experimental resins containing the zinc-doped polycarboxylated fillers (β-CS-Zn; BAG-Zn) had no significant drop in FT after 30 days of AS storage ($p>0.05$). The mean FT was affected by the material type ($F = 79.61$; $P < 0.001$), and by storage time ($F = 26.5$; $P < 0.001$).

3.4 Bioactivity and alkalinizing activity (pH)

The Raman cluster spectra of each tested material obtained at 24 h and after 30 d of SBF storage are illustrated in Figures 1 (A-H). It was observed that all the experimental resins containing the bioactive fillers presented, after cluster analysis, a prominent peak at 961 cm^{-1} after 30 d of AS storage, which indicated the formation of apatite. Conversely, the representative cluster of remineralization was never observed in specimens after 24 h. These results were confirmed during the SEM assessment of the specimens after 30 days of SBF storage. Presence of apatite was detected in all the resins containing bioactive fillers. However, differences in apatite crystal morphology were observed between the resins containing the ZnO/polycarboxylated fillers (BAG-Zn and β-CS-Zn) and those containing BAG

or β-TCS. No defined crystal structure was observed in the RES group. The specimens containing BAG and β-CS presented consistent precipitation of needle-like apatite crystallites (Fig. 1I). The resins BAG-Zn and β-CS-Zn showed precipitation of crystallites of apatite with a globular-like structure (Fig. 1L).

The mean and standard deviation of pH values attained during the alkalinizing activity evaluation are illustrated in Fig. 2G. The resins containing the bioactive micro-fillers BAG or β-CS induced an increase of the pH of the storage solution at 15 d and 30 d. However, the β-CS resin showed the most alkalinizing potential with a pH of 12.6 (±0.3) at 15 d and 12.7 (±0.2) after 30 d of storage in H_2O. The resin containing BAG filler had slight alkalizing potential (pH ~ 10.0). Conversely, the resins containing the ZnO/polycarboxylated fillers (BAG-Zn and β-CS-Zn) presented a more attenuated alkalinizing activity both at 15 d (pH ~ 8.6/8.8) and at 30 d (pH ~ 9/9.1). The resin control (RES) presented no change at 15 d (pH 6.7±0.2), but this material induced slight acidification of the media after 30 d of storage in H_2O (pH 5.7±0.3).

3.2 MTT Biocompatibility and Live & Dead assay

The mean and standard deviation of the results obtained with MTT are presented in Table 2, while the qualitative results Live & Dead are depicted in Figure 2. In brief, all experimental resins led to a significant viability decrease ($p<0.01$) compared to the cell control. However, filler-free resin (RES; Figure 2A) and β-CS resin (Figure 2B) led to cell death ($p<0.05$). Cells had a spindle-like morphology during Live&Dead fluorescence microscopy, which is indicative of cell death. Similar cell density and aspect was encountered for groups, BAG (Figure 2C) and the two zinc-doped fillers BAG-ZN and β-CS-Zn resin (Figure 2D).

3.3 Gene Expression & Mineralization

The mean and standard deviation of the results obtained with the gene expression assay are presented in Table 2. It was observed that MSCs grown in presence of the experimental materials differentiated into osteoblasts. Changes in markers of osteogenesis were analyzed by RT-qPCR and accumulation of mineralized matrix was visualized by alizarin red staining. MSCs differentiated in the presence of BAG showed slight effect on expression of either osteogenic markers, Runx-2 and ALP. Similarly, β-CS and control RES had no significant effect ($P>0.01$) on expression of osteogenic master switch (Runx-2) and ALP expression. Interestingly, incorporation of Zn to either BAG or β-CS had potent osteogenic effect with significant increase in expression of both markers ($P>0.01$). Runx-2 expression was increased by more than five fold ($p<0.05$) in both BAG-Zn and β-CS-Zn and ALP level by more than four-fold ($p<0.05$) when compared to cells cultured in presence of pure resin alone. The effect seen on mRNA expression of osteogenic genes by BAG-Zn (Fig. 2E) and β-CS-Zn (Fig. 2F) was also confirmed by the alizarin staining of calcium deposits.

4. DISCUSSION

Pulp protection materials used in the atraumatic restorative treatment (ART) technique should be able to evoke remineralization with no inflammatory pulp response [9-13]. Accordingly, this study aimed at generating advanced light-curable resin-based materials containing different types of experimental bioactive micro-fillers: evaluating their flexural strength, fracture toughness, biocompatibility, cell differentiation and bioactivity after storage in simulated body fluid (SBF). However, it is important to anticipate that the experimental light-curable resin-based materials with bioactive micro-fillers assessed in this study have not been developed to be used as direct pulp-capping agents.

The flexural strength (FS) is representative of tensions developed in the dental cavities, as it encompasses tensile, compressive and shear stresses under bending loads. Although FS is a static single-movement mechanical test, it is usually the first choice to survey the preliminary physical properties of newly developed resin-based materials. It is well known that several internal and superficial defects/imperfections may be present in specimens and materials [3], even when they seem flat and smooth to the naked eye. Therefore, under masticatory forces, materials undergo not only flexural tensions, but also the need to resist fracture/crack propagation; this measure is known as fracture toughness (FT). In view of the results obtained with the present FS and FT tests, the first null hypothesis that the addition of bioactive micro-fillers would have no effect on mechanical properties of the experimental resin-based materials tested must be partially rejected. Indeed, this study showed that there was no significant difference in FS and FT at 24 h between the resin-based materials containing the experimental micro-fillers and the control filler-free resin. However, all the tested experimental materials showed a significant drop in FS after 30 d storage of SBF storage, while the specimens created with the filler-free resin (RES) showed no significant reduction.

A possible explanation for the FS results obtained with the specimens created using the resin-based materials containing the experimental micro-fillers is that such a reduction may be attributed to the greater hydrophilicity of the included fillers, which absorb quantities of water [20] to evoke the bioactive processes for mineral precipitation (Fig 1 A-H). However, although these types of bioactive resin-based materials may not be suitable as definitive restorative materials due to their higher water sorption and solubility compared to conventional dental resin composites, they can be used as a pulp protection or temporary therapeutic materials in the stepwise restorative technique. [20]

The FT results showed a significant drop after 30 d storage of SBF storage only in the specimens created using the experimental BAG and β-CS and the filler-free resins. Conversely, the experimental resins β-CS-Zn; BAG-Zn showed no significant FT reduction (Table 1).

Such a drop in mechanical properties observed after prolonged aging in SBF (30d) can be due to the high opacity (low reflection index) of some of the micro-fillers (40 vol%) used in this study. These may have interfered with the photo-polymerization due to an incomplete diffusion of the light (energy) throughout the bulk of material. Such a phenomenon was potentially responsible for the decrease of monomer conversion, which caused an accelerated degradation of the resin matrix over time, especially in the experimental BAG and β-CS resins [26]. Conversely, in the filler-free control resin, the reduction of fracture toughness may be attributed to the hydrolytic degradation upon SBF immersion, with hydrolysis and elution of the more linear polymer chains within the resin matrix [27].

The experimental micro-fillers, β-CS-Zn and BAG-Zn, may have trigged a prolonged cationic polymerization within the polymer matrix. It is known that a cationic polymerization may be activated by polyionic silicates which form during the formulation of the BAG-Zn and β-CS–Zn micro-fillers treated with the polycarboxylic acidic solution [20, 28, 29]. Moreover, it has been stated that glass polyalkenoate cements containing zinc oxide may react with PAA and set via a two-step setting mechanism: 1) zinc-polyacrylate species cause primary hardening of the cement and later; 2) during water storage, formation of calcium-polyacrylate may induce a second post-hardening step. Furthermore, calcium ions released from the experimental micro-fillers may have reacted with the carboxylate group in the polyacrylic acid and stabilized the mechanical properties resin BAG-Zn and βTCS-Zn during prolonged AS storage [30]. However, it is also likely that bioactive ion leaching and the bioactive transformation of the fillers into needle-like apatite (Fig. 2I) at a strongly alkaline pH (Fig 2G) may have created

excessive micro-porosities within the experimental BAG and β-CS resins. This may have caused a significant reduction in FT over time [20]. The experimental BAG-Zn and β-CS-Zn resins showed globular-like apatite precipitation (Fig. 2L) at mildly alkaline pH (Fig. 2G). In this case, it is hypothesized that the reduction in FT was less evident due to the fewer porosities within the material created by a more compact mineral precipitation. All the results described so far seem to be in accordance with those previously reported by Sauro *et al.*, (2013) [20] and by Le *et al.*, [31]. The latter team demonstrated that, in a strongly alkaline environment, (pH 10–11) apatite precipitates with a morphology characterized by clusters of acicular crystals (needle-like structures). At a mildly alkaline pH (8–9), apatite precipitates as globules of nano-particles (globular-like structure) due to a fast nucleation rate so that more particles are formed and the nuclei of crystals grow slower due to the low temperature [32].

It is important to consider that the differences observed in these experimental resins in terms of pH, kinetics of precipitation and morphology of the apatite may have influenced the biocompatibility and played a role in the cell growth/differentiation. Indeed, the present results showed that all the resin-based materials tested led to a significant cell viability decrease (MTT test) compared to the negative control where the cells were seeded in the absence of resin-based material. However, the filler-free resin (RES) and β-CS resin induced cytotoxicity and the cells showed a spindle-like morphology, indicative of cell death (Figure 2A and 2B). The effects of the tested materials on the mineralization potential were investigated at the mRNA level, enzymatic level (ALP only) and extracellular matrix mineralization level (Alizarin red staining). The RES and β-CS showed no significant effect on the expression of the osteogenic markers, Runx-2 and ALP. All these outcomes encountered with the β-CS resin may have been a consequence of the ability of such material to make the medium alkaline over time (Fig 2G) [33]. Moreover, it is speculated that there may have been

an important elution of unreacted monomers in all resins [27]. This led to a significant cell viability reduction compared to the control (no resin material). Previously, the possibility of a low degree of conversion in the β-CS due to a lack of photo-polymerization was discussed. In this case, it may have caused the elution of unreacted monomers and initiator/co-initiator agents causing greater cytotoxicity with the β-CS resin [34, 35]. Leaching of such components may accumulate over time to a toxic level, which deplete irreversibly the defense mechanisms of cells and result in cell apoptosis [36]. The β-CS resin has a composition very similar to that of a commercial pulp protection material known as Theracal LC (containing 40-50 wt% of Portland cement powder, 30-40 wt% of neutral or mildly acidic hydrophilic resin monomers, 5-10 wt% of a hydrophobic resin monomer (BisGMA) and 5-10 wt% of a hydrophilic filler). Indeed, the present results seem to be in accordance with those of Bortoluzzi *et al.*, [33], who showed that Theracal LC caused a reduction in cell metabolism and protein expression when in contact with pulp cells. However, apart from β-CS resin, the drop in absorbance (MTT) was less than 50% compared to the control cells groups, which may represent minor (reversible) cytotoxic effects [37].

Similar cell density and morphology were encountered for resin containing BAG (Figure 2C) and those containing the zinc-doped polycarboxylate fillers (BAG-ZN and β-CS-Zn) (Figure 2D). These materials showed less cytotoxicity compared to the control RES and β-CS. Moreover, the resins BAG-Zn and β-CS-Zn were able to induce an osteogenic effect with significant increase in expression of both markers (Runx-2 and ALP). Calcium deposits were observed using the alizarin staining technique in the BAG-Zn (Fig. 2E) and β-CS-Zn (Fig. 2F) resins.

In view of these results it is possible to confirm that the second null hypothesis, the addition of bioactive micro-fillers would cause increase in cytotoxicity and induce no differentiation in primary human mesenchymal stem cells (MSCs), should be partially rejected as these factors

are associated with the chemical properties and composition of each single tested bioactive filler.

Recently, Ca–Si-based ceramics doped with specific therapeutic elements (Mg [38], Zn [39], and Sr [40]) have been designed to improve their biological properties. In particular, Zn has been demonstrated to have a stimulatory effect on bone formation and an inhibitory effect on osteoclastic bone resorption [41]. Indeed, Zn ions appear to induce ALP activity, since ALP is a Zn-dependent enzyme [42]. Zinc deficiency induces arrest of bone growth, bone development, and jeopardizes the overall maintenance of bone health [43, 44]. Yu *et al.*, [45] showed that bioceramics doped with zinc (e.g. $Ca_2ZnSi_2O_7$) enhances attachment, proliferation, differentiation and up-regulated bone marker gene expression of ALP and growth factor genes (e.g. IGF-I and TGF-β1), compared to zinc-free silicates ($CaSiO_3$). Chesters [46] and Cousins [46] reported that zinc is a crucial trace element that induces several metabolic, cellular signaling pathways and gene expression for bone formation. Moreover, the incorporation of zinc into calcium phosphate cement significantly promotes pre-osteoblast proliferation and differentiation *in vitro* [47-50]. Furthermore, it was also suggested that zinc is involved in regulating the transcription of pre-osteoblast differentiation genes (e.g. Col-I, ALP and osteopontin) [51]. Hence, zinc is considered a promising agent for enhancing the bone-forming ability of implant materials, which can be achieved by controlling the release of Zn ions.

5. CONCLUSIONS

The present study has demonstrated that the incorporation of the experimental fillers into resin-based materials makes them “bioactive” enough to induce apatite precipitation. However, only the resins containing the two zinc-doped bioactive fillers are able to maintain

stable mechanical properties over time. In terms of biocompatibility and osteo-inductivity/dentinogenesis, the resins containing the zinc-doped fillers (β-CS-Zn and BAG-Zn) were the most promising as possible alternative pulp protection materials for use clinically.

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FIGURES CAPTION

FIGURE 1: Raman spectroscopic cluster analysis of principal components identified before and after storage [resin and remineralization]. The resin BAG at 24 h showed a Raman resin cluster with no sign of Ca/P minerals on the outer surface (A), while after 30 days of SBF storage, it was able to induce apatite precipitation (note the peak at 961 cm^{-1}) (B).

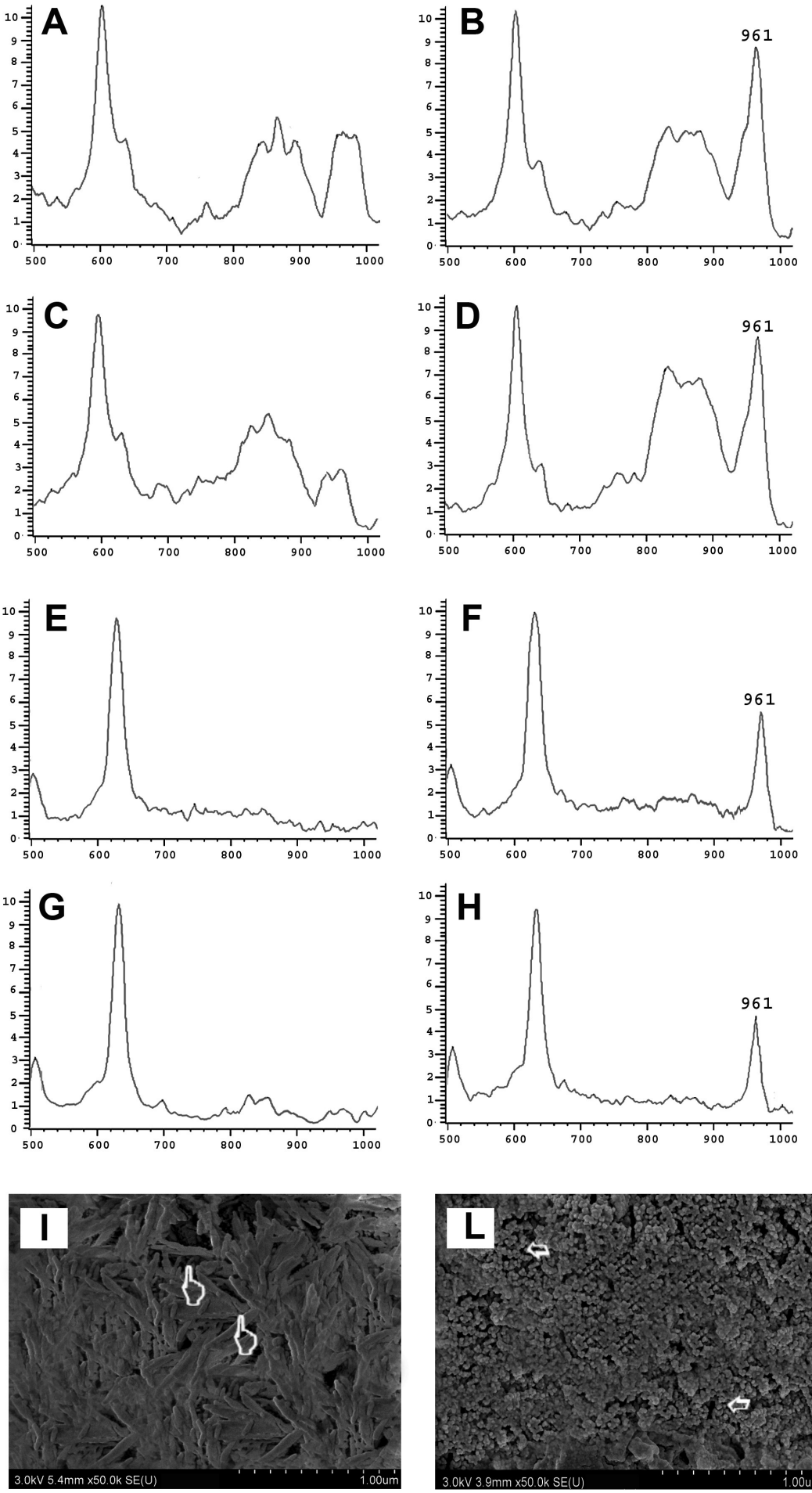
The same situation was observed with the resin BAG-Zn at 24 h, which showed no sign mineralization (C), but clear apatite precipitation (note the peak at 961 cm^{-1}) after 30 days of SBF storage (D).

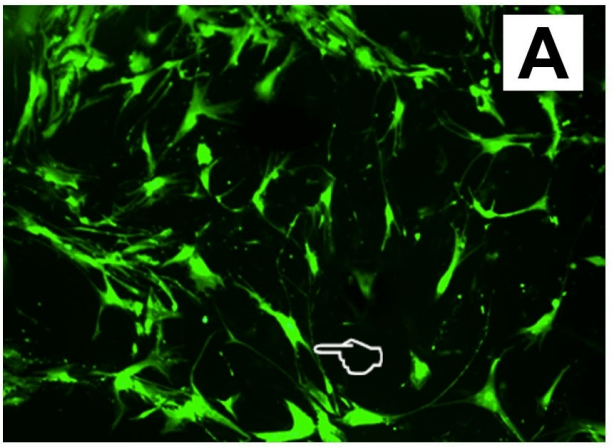
The resin β-CS (E) and the β-CS-Zn (G) showed at 24 h showed only a Raman cluster with no sign of mineralization. These two resins β-CS (F) and the β-CS-Zn (H) were able to induce apatite formation (note the peak at 961 cm^{-1}) after 30 days of SBF storage. Figures I and L are representative SEM micrographs showing respectively a needle-like apatite formation (pointer) in resins containing filler that were not doped with zinc (BAG and β-CS) and globular-like nano-apatite formation (arrow) in resins containing the zinc-doped fillers (BAG-Zn and β-CS-Zn).

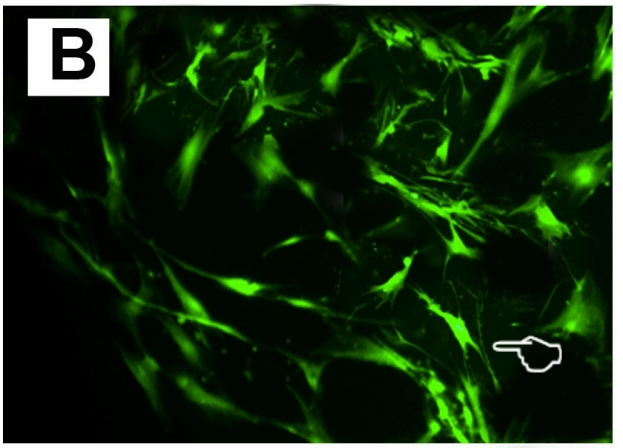
FIGURE 2: Images obtained during the Live&Dead fluorescence microscopy. It is possible to see spindle-like cell morphology (pointer), which is indicative of cell death, in the group of the filler free resin (A) and β-CS resin (B).

The group BAG, BAG-ZN and β-CS-Zn presented similar cell density. Figure C is a representative image obtained in presence of the BAG and figure D is a representative image obtained with the resin containing the zinc-doped fillers (BAG-ZN and β-CS-Zn). In all cases, it is possible to see great cell density and a clear differentiation of the MSCs into fibroblasts (pointer).

Figure (E) and (F) are optical micrographs (alizarin red staining technique) that show the calcium deposits (dark areas) observed in the resins BAG-Zn and β-CS-Zn, respectively. In figure (G) it is reported the pH value of each material tested in this study. It is possible to see that the most alkalinizing resin was the β-CS, which was able to maintain a strong alkaline pH over time [pH > 12]. While the other experimental resins presented a milder pH, in particular those containing the zinc-doped fillers.


A
B
961
C
D
961
E
F
961
G
H
961
I
3.0kV 5.4mm x50.0k SE(U)
1.00um
L
3.0kV 3.9mm x50.0k SE(U)
1.00um


A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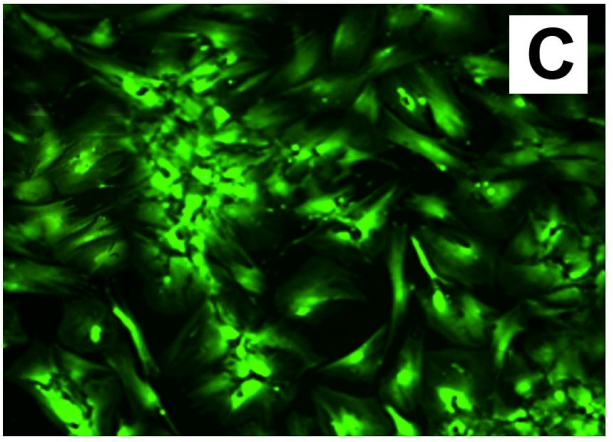
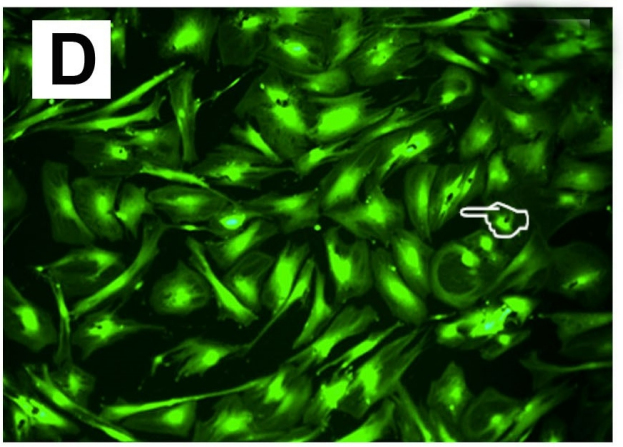

C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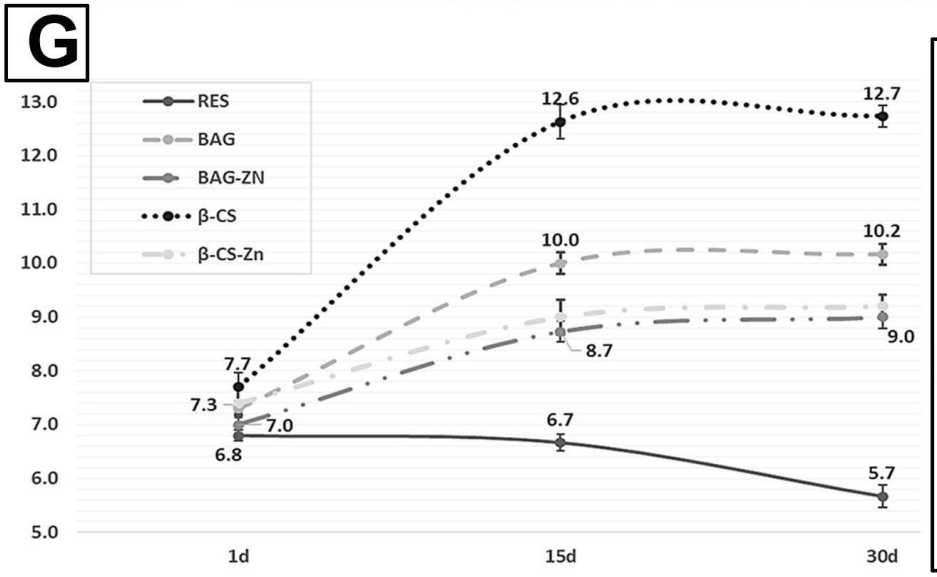
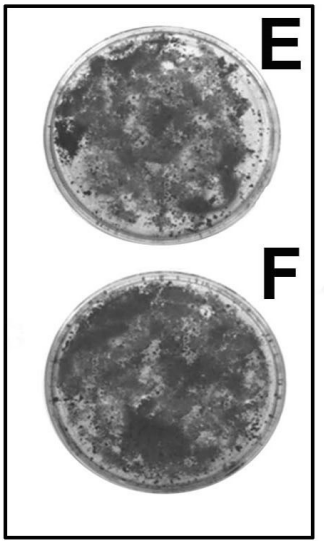

G
RES
BAG
BAG-ZN
β-CS
β-CS-Zn
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
1d
15d
30d
12.6
12.7
10.0
10.2
8.7
9.0
7.7
7.3
7.0
6.8
6.7
5.7


E
F

	Flexural strength (FS; MPa)		Fracture Toughness (FT; MPa.m^0.5)	
	24h	**30d**	**24h**	**30d**
RES	98.0±12.9 [A1]	99.2±11.2 [A1]	2.7±0.5 [A1]	2.0±0.2 [A2]
BAG	88.4±15.6 [A1]	48.0±5.6 [B2]	2.4±0.5 [A1]	1.2±0.2 [B2]
BAG-Zn	91.3±13.4 [A1]	53.1±9.4 [B2]	2.5±0.4 [A1]	1.9±0.2 [A1]
β-CS	85.3±16.4 [A1]	55.6±7.1 [B2]	1.9±0.4 [A1]	1.3±0.3 [B2]
β-CS-Zn	79.6±8.6 [A1]	50.9±8.4 [B2]	2.3±8.5 [A1]	2.1±0.1 [A1]

Table 1. Flexural Strength (FS) and Fracture Toughness (FT) of experimental resins after 24 h and 30 d ageing in AS at 37°C.

Similar uppercase letter indicates no significant differences in column at 24h or 30d. ($p>0.05$)

Similar number indicates no significant differences in row between 24h and 30d. ($p>0.05$)

	MTT		mRNA gene expression	
	14 days		**Runx2**	**ALP**
Cell - control	0.5±0.01 [A]		-	-
RES	0.2±0.03 [B]		1.1±0.1 [A]	1.0±0.1 [1]
BAG	0.4±0.04 [C]		1.74±0.5 [A]	1.3±0.3 [1]
BAG-Zn	0.4±0.04 [C]		4.9±1.7 [B]	4.1±1.2 [2]
β-CS	0.1±0.05 [D]		1.7±0.2 [A]	1.5±0.1 [1]
β-CS-Zn	0.4±0.05 [C]		4.8±1.9 [B]	4.7±1.9 [2]

Table 2. Biocompatibility (MTT) and Cell differentiation (Gene expression Runx2 and ALP) of experimental resins after 24 h and 30 d ageing in AS at 37°C.

Similar uppercase letter indicates no significant differences in column for MTT and Runx2 ($p>0.01$)

Similar number indicates no significant differences in column for ALP ($p>0.001$).