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Development and characterization of a new chitosan-based scaffold associated with gelatin, microparticulate dentin and genipin for endodontic regeneration

Hector Caballero-Flores[a,*], *Cleber Keiti Nabeshima*[a], *Giovanna Sarra*[a], *Maria Stella Moreira*[b], *Victor Elias Arana-Chavez*[c], *Márcia Martins Marques*[b], *Manoel Eduardo de Lima Machado*[a]

[a] *Discipline of Endodontic, Department of Restorative Dentistry, School of Dentistry, University of São Paulo, São Paulo, SP, Brazil*

[b] *Post Graduation Program, School of Dentistry, Ibirapuera University, São Paulo, SP, Brazil*

[c] *Department of Biomaterials and Oral Biology, School of Dentistry, University of São Paulo. São Paulo, SP, Brazil*

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ABSTRACT

Objective. An ideal scaffold for endodontic regeneration should allow the predictableness of the new tissue organization and limit the negative impact of residual bacteria. Therefore, composition and functionalization of the scaffold play an important role in tissue bioengineering. The objective of this study was to assess the morphological, physicochemical, biological and antimicrobial properties of a new solid chitosan-based scaffold associated with gelatin, microparticulate dentin and genipin.

Methods. Scaffolds based on chitosan (Ch); chitosan associated with gelatin and genipin (ChGG); and chitosan associated with gelatin, microparticulate dentin and genipin (ChGDG) were prepared by using the freeze-drying method. The morphology of the scaffolds was analyzed by scanning electron microscopy (SEM). The physicochemical properties were assessed for biodegradation, swelling and total released proteins. The biological aspects of the scaffolds were assessed using human cells from the apical papilla (hCAPs). Cell morphology and adhesion to the scaffolds were evaluated by SEM, cytotoxicity and cell proliferation by MTT reduction-assay. Cell differentiation in scaffolds was assessed by using alizarin red assay. The antimicrobial effect of the scaffolds was evaluated by using the bacterial culture method, and bacterial adhesion to the scaffolds was observed by SEM.

Results. All the scaffolds presented porous structures. The ChCDG had more protein release, adhesion, proliferation and differentiation of hCAPs, and bacteriostatic effect on *Enterococcus faecalis* than Ch and ChGG ($p < 0.05$).

* *Corresponding author at*: Department of Restorative Dentistry, School of Dentistry, University of São Paulo, Av. Prof. Lineu Prestes, 2227, Butantã, São Paulo, SP, 05508-000, Brazil.
E-mail address: h.caballero-flores@usp.br (H. Caballero-Flores).

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Significance. The chitosan associated with gelatin, microparticulate dentin and genipin has morphological, physicochemical, biological and antibacterial characteristics suitable for their potential use as scaffold in regenerative endodontics.

1. Introduction

The regenerative endodontics proposes to replace damaged structures of the pulp-dentin complex by means of tissue engineering. Growth factors signal the cell differentiation within the scaffold, which in turn should favor the cell activity [1]. However, the generation of suitable microenvironment for regeneration of pulp-dentin complex is a challenger due to the difficult of control stem cell fate, limited blood supply and, in necrotic tooth a presence of residual bacterial [2–5].

Chitosan is a natural polymer used for preparation of scaffolds due to for ability to form highly biocompatible porous structures with the ability to regulate the release of bioactive agents [6,7]. However, pure chitosan scaffold limit suitable adhesion of human dental pulp stem cells [8,9]. Therefore, the addition of compounds may be necessary to improve cell behavior [9–11]. Regard to endodontic regeneration, Palma et al. [12] reported that the addition of chitosan/hyaluronic acid or chitosan/pectin scaffolds to blood in regenerative procedures in dogs did not improve the histologic evidence of the regeneration of a pulp-dentin complex. Additionally, the supplemented of fibrin scaffold with chitosan did not inhibit the bacterial grown [13]. Thus, optimization of chitosan-based scaffolds is clearly required to improved biological and antibacterial properties.

In this sense, gelatin is a biodegradable, biocompatible, low-immunogenic material favoring adhesion and migration of cells [14,15], whereas genipin is a natural cross-linking agent that promotes odontogenic differentiation [16]. The use of these compounds in a chitosan-based scaffold has not been studied yet in regenerative endodontics. Functionalization of scaffolds is challenging because various growth factors are involved to control cell fate, and the adequate amount of them is still unknown [17]. Studies have shown that dentin has growth factors that favors migration, proliferation and differentiation of cells [18,19]. Additionally, peptides in the dentin matrix and peptides derived from HB-EGF, amphiregulin, hepatocyte growth factor, PDGF-A, PDGF-B and FGFs growth factors have showed antimicrobial activity on gram positive and negative bacteria [20,21]. Therefore, functionalization of scaffolds by using dentin could create a suitable microenvironment for tissue regeneration avoided the use of recombinant growth factor and/or chemical substances for bacteria control.

The objective of this study was to assess the morphological, physicochemical, biological and antimicrobial characteristics of a new solid chitosan-based scaffold associated with gelatin, microparticulate dentin and genipin.

2. Material and methods

Low molecular weight chitosan from shrimp shells (75–85% deacetylation degree, 50–190 KDa), type B bovine skin gelatin and the others chemicals used were purchased from Sigma-Aldrich (Saint-Louis, USA), unless noted otherwise.

2.1. Development of the scaffolds

Scaffolds based on chitosan associated with gelatin and microparticulate dentin, cross-linked with genipin (ChGDG), were prepared as follows: dentin particles (0.3–53 μm, Appendix A) were obtained from crowns of human molars, added with acetic acid 1% (w/v), ultrasonicated and agitated for 4 h. Chitosan 1% (w/v) was added and agitated for 24 h, followed by addition of gelatin 2% (w/v) and agitation for 2 h, and cross-linked by addition of genipin 0.15% (w/v) and agitation for 30 min. The final solution was ultrasonicated for 10 min and lyophilized. The scaffolds were neutralized in anhydrous ethyl alcohol for 12 h and lyophilized.

Scaffolds based on chitosan associated with gelatin, cross-linked with genipin (ChGG), were prepared by using solution of chitosan 1% (w/v) and gelatin 2% (w/v), both cross-linked with genipin 0.15% (w/v) in acetic acid 1% (w/v).

Scaffolds based on pure chitosan (Ch) were prepared by using chitosan 2% (w/v) in acetic acid 1% (w/v).

All the scaffolds were sterilized by gamma radiation.

2.2. Morphological characterization

The scaffolds (n = 4/group) were frozen in liquid nitrogen, transversally cleaved and sputter coated with a gold layer for analysis using a scanning electron microscope (JEOL, Massachusetts, USA). The diameter of the pores was measured in the scanning electron micrographs using the ImageJ software (Maryland, USA).

2.3. Biodegradation assay

Each scaffold (n = 4/group) was weighted (Wo) and immersed into 2.144 ml of simulated fluid body (SFB) [22], according to ISO 10993-13 [23] for 2, 8, 16, 24 and 32 days, and 1.5 μg/mL of lysozyme was added daily before they were lyophilized and weighed again (Wt). Biodegradation rate (D%) was determined according to the formula [24]: $D\% = Wo - Wt/Wo \times 100$.

The 32-day samples were processed for assessment of the structure of the scaffolds by SEM.

Table 1 – Antimicrobial properties of the chitosan-based scaffolds. Median (Q1-Q3) of bacterial grown in presence of the scaffolds.

	Bacteria concentration (CFU/mL)			
	C	Ch	ChGG	ChGDG
0 h	1×10^5 ∞, a (0.6×10^5–2.8×10^5)	1×10^5 ∞,a (0.6×10^5–2.8×10^5)	1×10^5 ∞,a (0.6×10^5–2.8×10^5)	1×10^5 ∞,a (0.6×10^5–2.8×10^5)
6 h	1.8×10^8 €, a (1.6×10^8–2.0×10^8)	1.1×10^5 ∞, b (7.6×10^4–1.6×10^5)	3.2×10^7 €, c (1.6×10^7–3.6×10^7)	7.8×10^6 €, c (2.4×10^6–1.4×10^7)
24 h	3.7×10^8 £, a (3.1×10^8–3.9×10^8)	1.2×10^7 €, a (7.4×10^6–1.6×10^7)	9.9×10^7 €, b (3.2×10^7–$1,7 \times 10^8$)	5.5×10^6 €, b (4.2×10^6–1.1×10^8)
48 h	4.8×10^7 €, a (2.5×10^7–1.0×10^8)	4.9×10^6 €, a (4.0×10^6–6.2×10^6)	6.2×10^7 £, b (2.8×10^7–8.5×10^7)	4.0×10^5 ∞, c (3.3×10^5–9.4×10^5)

Different symbols indicate intragroup significant differences and different letters indicate intergroup significant differences ($p < 0.05$). C, control; Ch, pure chitosan scaffold; ChGG, Chitosan-gelatin-genipin scaffold; ChGDG, Chitosan-gelatin-microparticulate dentin-genipin scaffold.

2.4. *Swelling assay*

Each scaffold (n = 4/group) was weighted (Wd) and incubated for 4 and 8 h, as described earlier, before being dried and weighted again (Ws). The swelling rate (S%) was calculated according to the formula [24]: S% = Ws − Wd/Wd × 100.

2.5. *Total protein released assay*

Each scaffold (n = 4/group) was immersed into 2.144 mL of phosphate buffered saline (PBS; pH = 7.4) [25]. and incubated for 1, 2, 8, 16, 24, and 32 days. Then, 20 μL of supernatant was collected and stored at – 80 °C, and the amount of total protein released was assessed by the Lowry method [26]. Scaffold with no addition of PBS was used as control.

2.6. *Isolation and cell characterization*

Details of isolation and immunophenotype characterization of human cells from apical papilla (hCAPs) are described in Appendix B.

2.7. *Direct cytotoxicity assay*

The scaffolds (n = 4/group) were submitted to direct contact test [27]: the samples were immersed into clonogenic cell culture medium (alpha-MEM supplemented with 15% of bovine fetal serum, 100 μM of ascorbic acid, 2 mM of L-glutamine, 100 U/mL of penicillin and 100 μL of streptomycin) during 4 h and then, the hCAPs were plated (1×10^3 cells) on the top of the scaffolds. The scaffold interface was observed with a phase inverted microscope (Olympus IX71, Tokyo, Japan) on days 2 and 8. Scaffolds with no hCAPs served as control.

2.8. *Indirect cytotoxicity assay*

The scaffolds were immersed into clonogenic medium for 24 h for obtaining the conditioned media [25]. These conditioned media were added to hCAPs (1×10^3 cells, n = 8/group) and incubated for 24 or 48 h [27]. Then, the cell viability was analyzed by MTT test. Cells cultured in clonogenic medium served as control.

2.9. *Cell adhesion and proliferation*

The scaffolds (n = 20/group) (4 × 4 mm) were individually adapted to inside polytetrafluorethylene tubes, which were sealed at the bottom with a cellulose-based membrane (Merck Millipore, Darmstadt, Germany) before sterilization with gamma radiation. This setting was made for simulate a closed environment of the root canal but free of the dentin. hCAPs of passage 3 were plated on the top of the scaffolds (5×10^4 cells/scaffold) and after 3 h the scaffold-tube set (n = 8/group) was transferred to another cell culture plate and incubated for 4 or 8 days. Next, the scaffold-tube set was transferred to another plate for the MTT test.

Two scaffolds from each group after 4 and 8 days were processed and observed by SEM for analysis of cell morphology and adhesion.

Cells cultured on polystyrene discs served as positive control, whereas scaffolds with no cells served as negative control.

2.10. *Cell differentiation*

Inoculation of hCAPs in the scaffolds (n = 8/group) was performed as described in Section 2.12 and incubation in clonogenic medium was done for 7 days. Next, this medium was replaced by a mineralizing medium (alpha-MEM supplemented with 15% of bovine fetal serum, 100 U/mL of penicillin, 100 μL of streptomycin, 2 mM of L-glutamine, 100 μM of ascorbic acid, 1.8 mM potassium phosphate and 10 μL of dexamethasone) before incubation for 21 days. Formation of mineralized nodules was evaluated by using the Alizarin red test. The scaffolds with no cells were used to eliminate the background.

Cells cultured on polystyrene discs in mineralizing medium served as positive controls, whereas those in clonogenic medium served as negative controls.

2.11. *Antimicrobial testing*

Cylinder scaffold (4 × 5 mm) were individually adapted to inside well of 96 culture plaque. Each scaffold (n = 8/group) and an aliquot of 200 μL of *Enterococcus faecalis* (ATCC 29212) (1×10^5 CFU/mL) were incubated for 48 h. An aliquot of 10 μL was collected after 6, 24 and 48 h, diluted (10-fold) and plated on m-Enterococcus agar in triplicate for CFU counting. Scaffolds

(n = 2/group) were processed and observed by SEM. Bacteria cultured on nitrocellulose membrane served as control.

Additionally, the scaffolds (n = 8/group) were incubated in 1 mL of saline solution for 8 h. Next, an aliquot of 100 μL was added to 100 μL of bacterial suspension (1×10^5 CFU/mL), incubated, plated and assessed as earlier described. The non-conditioned serum added to the bacterial suspension served as control.

2.12. Statistical analysis

ANOVA and Tukey's post hoc test were used to intragroup analysis of pore size, biodegradation, swelling, protein release, indirect cytotoxicity, cell proliferation and cell differentiation. Repeated measurement ANOVA and paired t-test adjusted by the Bonferroni method were used to intragroup analysis of the protein release. T test was used to intragroup analysis of the indirect contact assay and cells proliferation. The inter-group analysis of the antibacterial activity was assessed by using Friedman and Nemenyi tests with correction of paired data, and intragroup by using Kruskal–Wallis and Dunn test. All statistical analyses were performed at a significant level of 5%.

3. Results

3.1. Morphological characterization

All the scaffolds exhibited interconnecting pores (Fig. 1F, H and J). Microparticulate dentin were homogeneously distributed, resulting in a rough surface with small pores (Fig. 1A).

3.2. Biodegradation and swelling

The rates of biodegradation (Fig. 1B) and swelling (Fig. 1C) in groups ChGG and ChGDG were similar, but lower than those in group Ch in all experimental times. Scanning electron micrographs showed that scaffolds in group Ch had structure collapsed, whereas those in group CGG had no alteration and those in group ChGDG had greater pore interconnectivity after biodegradation assay (Figs. 1G, I, and K).

3.3. Total protein released

Groups Ch and ChGDG showed lower and higher release of proteins, respectively (Fig. 1D). The comparison between experimental times showed that the group C had maximum protein release on day 2, with no difference compared to day 1, whereas those in groups ChGG and ChGDG had an increase up to day 8 (Fig. 1E). The control group showed absence of protein in all the experimental times.

3.4. Cell characterization and cytotoxicity assay

The isolated cells expressed mesenchymal stem cell surface markers with immunophenotypic characteristics of fibroblastic/stem/stromal cells (Fig. 3A).

With regard to the direct contact, the phase photomicrographs showed growth and contact with the scaffolds in all groups (Fig. 2E–H).

Conditioned medium of the group Ch showed lower cell growth, without difference between the 24-h and 48-h periods. However, conditioned medium of the group ChGDG showed greater cell growth compared to the other groups and experimental times (Fig. 2I).

None of the conditioned media reduced the cell viability at levels lower than 60%. Cell viability was higher in the group ChGDG for both experimental times (Fig. 2J and K).

3.5. Cell morphology, adhesion and proliferation

In group Ch, the surface of the scaffolds showed spherical cells up to day 8. In group ChGDG, these cells were observed on day 4 and some of them spread on day 8. Addition of microparticulate dentin resulted in cell spreading with filopodia attached to the scaffolds on day 4 and covering its entire surface on day 8 (Fig. 3E and EF).

In groups ChGG and ChGDG, the scaffolds showed greater cell viability on day 8 than on day 4, whereas those in group Ch showed similar cell viability in both experimental times. All groups differed between them in the fourth day, with scaffolds in group Ch and control showing lower and higher absorbance, respectively. The groups ChGDG and control were similar on day 8, with both showing higher absorbance than the other groups. Group Ch had the lower value on day 8 (Fig. 2O).

3.6. Cell differentiation

The ChGDG scaffold group presented mineralized nodules formation similar to that of control group, which was higher than the other groups (Fig. 2P and Q).

3.7. Antibacterial effect

The presence of scaffolds interfered with bacterial growth, with group Ch presenting lower CFU/mL on day 6. The number of bacteria was similar between the groups ChGG and ChGDG, but was greater than that of group Ch and control. In the 24-h period, scaffolds in groups ChGDG and C had similar antibacterial effects, but which were lower than those of ChGG and control. However, group ChGG and ChGDG had lower CFU/mL in the 48-h period, which was similar to that measured at the baseline (Table 1).

All groups were found to be similar regarding the contact of *E. faecalis* with leachable substances of scaffolds, since bacterial growth was observed up to 24 h. Nevertheless, there was a growth reduction of bacteria in ChGDG between 24 and 48 h. CFU/mL was lower in groups ChGDG and C after 6 and 24 h. Only group ChGDG showed a lower value after 48 h (Table 2).

Bacteria were adhered to all scaffolds, but those of group ChGDG were less likely to have such an adhesion (Fig. 4).

4. Discussion

The regeneration of endodontic tissue is challenging due to the difficulties in generating a suitable microenvironment

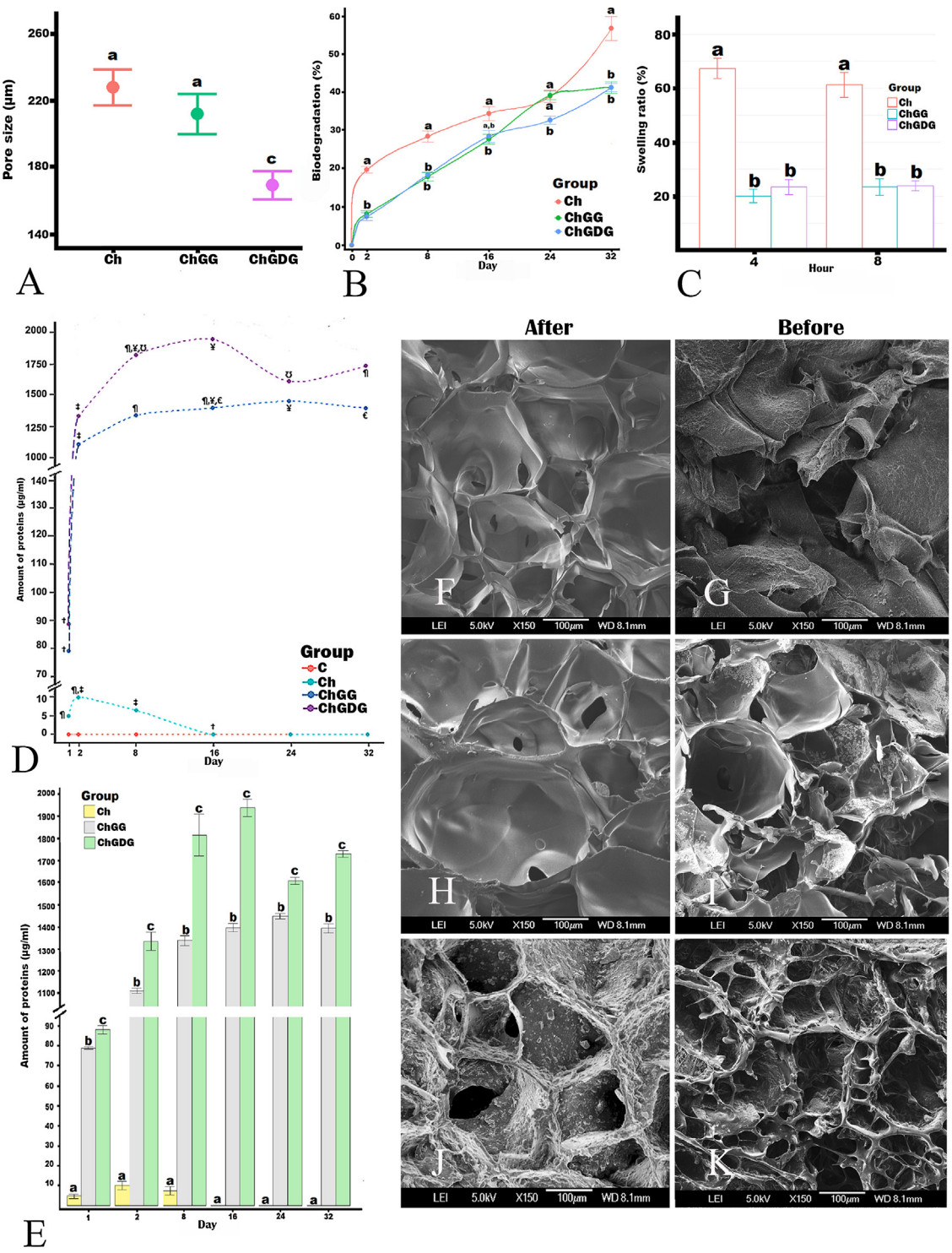

Fig. 1 – Morphological and physicochemical characterizations of chitosan-based scaffolds. (A) Pore sizes of scaffolds. (B) Biodegradation ratio of scaffolds. (C) Swelling ratio of scaffolds. (D) Intragroup comparison of the total protein released from scaffolds. (E) Intergroup comparison of the total protein released from scaffolds. For all assay each point or bar represents the mean ± SD (n = 4/group). Different letters or symbols indicate significant differences ($P < 0.05$). Representative SEM micrographs of scaffolds of Ch (F, G); ChGG (H, I) and ChGDG (J, K) before and after biodegradation assay. Scale bar 100 μm. Ch, pure chitosan scaffold; ChGG, Chitosan-gelatin-genipin scaffold; ChGDG, Chitosan-gelatin-microparticulate dentin-genipin scaffold; C, control.

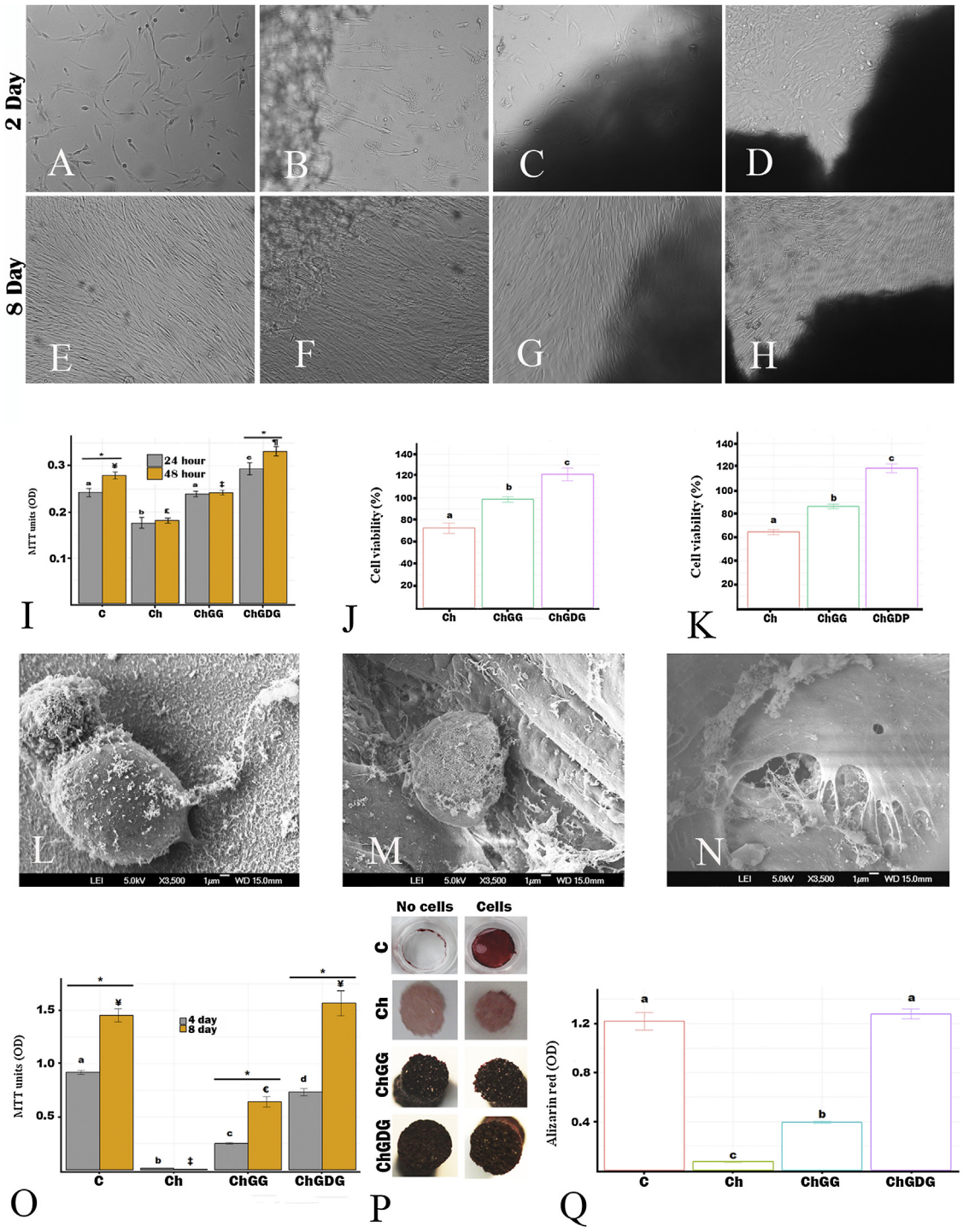

Fig. 2 – Biological characterization of chitosan-based scaffolds. Representative phase photomicrographs of direct contact assay of hCAPs only (A, E) or with scaffold of Ch (B, F), ChGG (C, G) and ChGDG (D, H) at two and eight days. (I) hCAPs viability in contact with leachable substances of scaffolds. (J, K) Relative hCAPs viability at 24 (J) and 48 h (K) in contact with leachable substances of scaffolds. Representative SEM micrographs of hCAPs cultured on scaffolds for 8-day. Spherical cell on scaffold of Ch (L), spherical cell and spreading cell (arrow) on scaffold of ChGG (M) and cell spreading on scaffold of ChGDG (N). Scale bar 1 μm. (O) Proliferation assay of hCAPs on scaffolds. (P) Representative images of scaffolds stained with alizarin red dye at the 28-day time point. (Q) Differentiation assay of hCAPs on scaffolds. For all assay each bar represents the mean ± SD (n = 8/group). Different letters or symbols indicate intergroup significant differences (P < 0.05). (*) indicates intragroup significant difference (P < 0.05). Ch, pure chitosan scaffold; ChGG, Chitosan-gelatin-genipin scaffold; ChGDG, Chitosan-gelatin-microparticulate dentin-genipin scaffold; C, control.

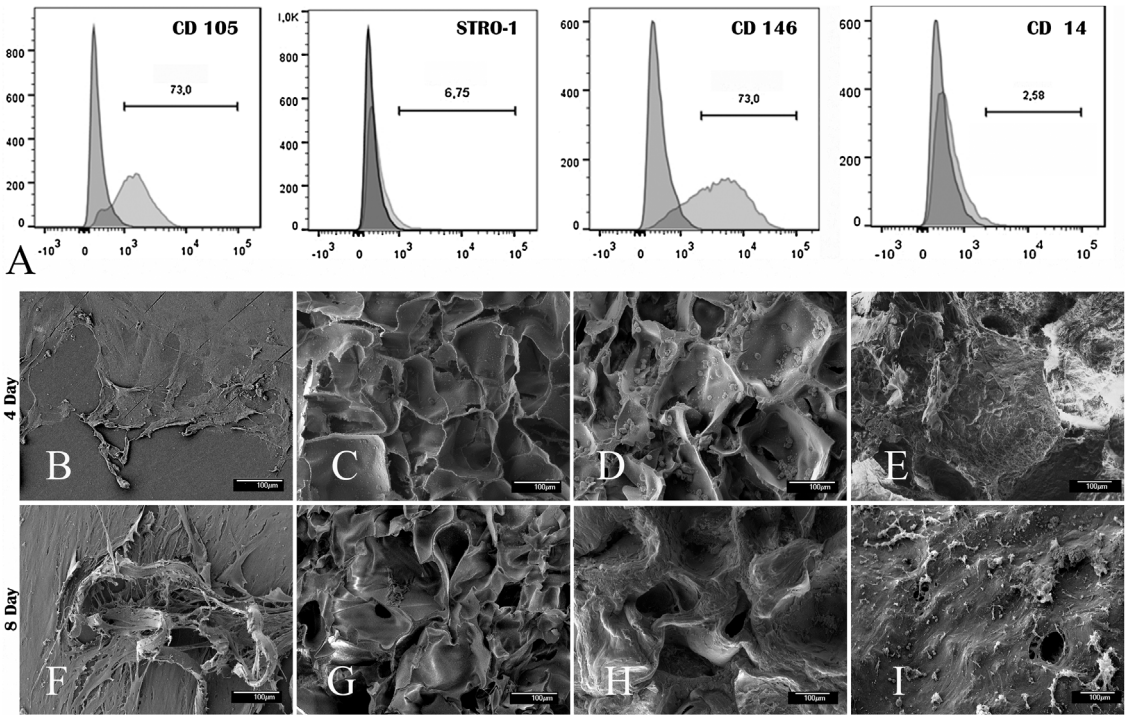

Fig. 3 – (A) Immunoprofile of the surface molecules of isolated cells. Positive for CD105, STRO-1 and CD146 and negative for CD14. Representative SEM micrographs of hCAPs cultured on polystyrene disc (B, F) or on scaffold of Ch (C, G), ChGG (D, H) and ChGDG (E, I) for 4 and 8-day. Scale bar 100 μm.

Table 2 – Antimicrobial properties of the chitosan-based scaffolds. Median (Q1-Q3) of bacterial grown in contact with leachable substances ofscaffolds.

	Bacteria concentration (CFU/mL)			
	C	Ch	ChGG	ChGDG
0 h	1×10^{5} ∞, a (0.6×10^{5}–1.8×10^{5})	1×10^{5} ∞,a (0.6×10^{5}–1.8×10^{5})	1×10^{5} ∞,a (0.6×10^{5}–1.8×10^{5})	1×10^{5} ∞,a (0.6×10^{5}–1.8×10^{5})
6 h	1.4×10^{7} €, a (9.8×10^{6}–1.9×10^{7})	3.7×10^{6} £, b (2.7×10^{6}–4.3×10^{6})	1.2×10^{7} £, a (7.0×10^{6}–3.1×10^{7})	2.5×10^{6} €, b (1.7×10^{6}–3.2×10^{6})
24 h	2.6×10^{8} £, a (2.2×10^{8}–2.1×10^{8})	7.6×10^{7} €, b (3.4×10^{7}–1.3×10^{8})	2.0×10^{8} €, a (1.8×10^{8}–2,5 $\times 10^{8}$)	1.7×10^{7} £, b (1.6×10^{7}–2.2×10^{7})
48 h	2.6×10^{8} £, a (1.7×10^{8}–3.3×10^{8})	1.6×10^{8} €, a (1.4×10^{8}–1.7×10^{8})	1.5×10^{8} €, a (1.5×10^{8}–1.6×10^{8})	2.3×10^{6} €, b 1.5×10^{6}–2.7×10^{6}

Different symbols indicate intragroup significant differences and different letters indicate intergroup significant differences ($p < 0.05$). C, control; Ch, pure chitosan scaffold; ChGG, Chitosan-gelatin-genipin scaffold; ChGDG, Chitosan-gelatin-microparticulate dentin-genipin scaffold.

for the proliferation of stem cells, for the proper control of their differentiation, also due to the limited blood supply and, in necrotic teeth, to a negative impact of bacteria residuals [2–5,28]. Therefore, several scaffolds have been proposed for regenerative endodontics, and the interaction between their components can interfere with their morphological, physicochemical and biological properties [29]. The present study assessed chitosan-based scaffolds and showed that the addition of gelatin and genipin favored the adhesion, proliferation and mineralization of hCAPs, as well as the addition of microparticulate dentin improved these biological characteristics and promoted antibacterial activity.

The regeneration of pulp-dentin complex comprises the combination and interplay of stem cells and/or progenitor cells, growth factors, and scaffolds [1]. These cells could be transplanted or mobilized by inducting their migration (cell homing) to the area to be regenerated or by establishing the bleeding of surrounding tissues, such the apical papilla [1,30–33]. The use endogenous cells is a clinical feasible approach, safer, less laborious and less expensive than transplanted cells approach [30,34]. Apical papilla is rich of resident cells of different degrees of differentiation in both vital and necrotic immature teeth [35,36]. We have worked with a pool of cells isolated from the apical papilla (hCAPs) most of them with stem cells markers, as observed by flow cytometry, to be closest as possible to a clinical situation of homing-based regenerative endodontics. In fact, hCAPs are widely used in endogenous cells strategy research for endodontic regeneration [34,37]. Additionally, the growth factors have a pivotal role to induced chemotaxis, proliferation

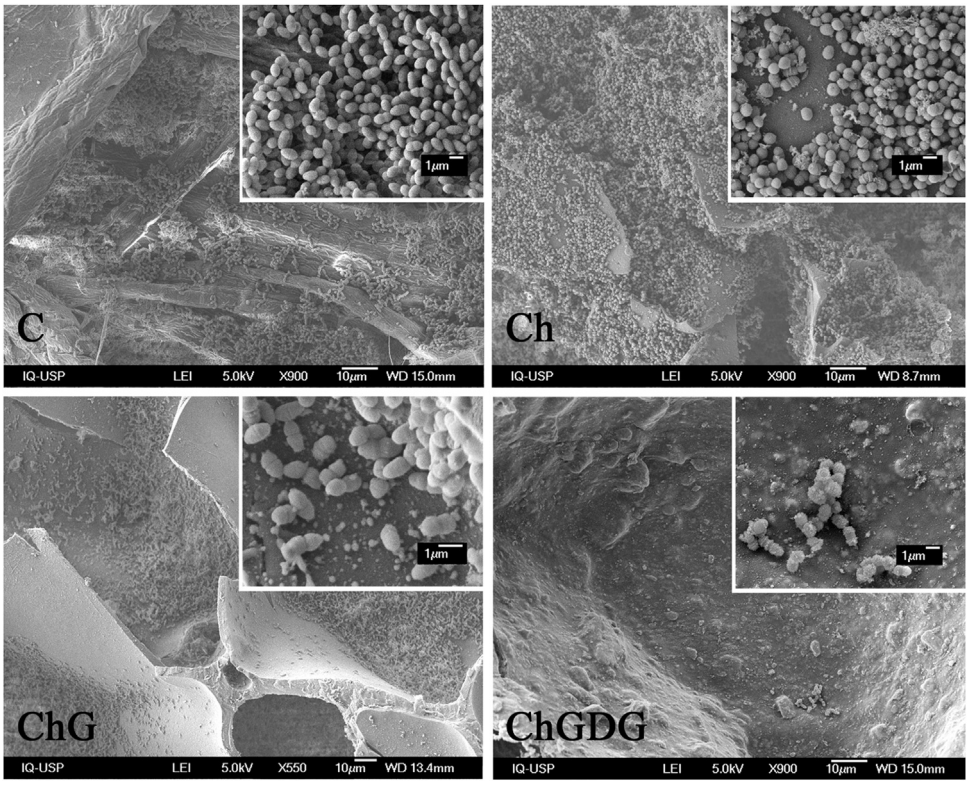

Fig. 4 – Representative SEM micrographs showing adhesion and proliferation of *E. Faecalis* cultured on cellulose membrane (C), or pure chitosan scaffold (Ch); or Chitosan-gelatin-genipin scaffold (ChGG); or Chitosan-gelatin-microparticulate dentin-genipin scaffold (ChGDG). Scale bar 10 μm.

and differentiation of dental stem cells [1,2,34]. The clinical use of recombinant growth factors is controversial because of the multiple morphogenic factors involved in the regeneration process, unknown ideal concentrations and possibility of adverse effects [2]. Therefore, endogenous acid soluble dentin proteins during endodontic treatment or incorporated into the scaffolds were proposed as an alternative to recombinant growth factors [18,19,34,37,38]. The combination of soluble endogenous dentin-derived growth factors has induced the chemotaxis, proliferation and differentiation of cells [37,38]. Moreover, Smith et al. [39] reported that insoluble dentin matrix improved the dentinogenesis activity of soluble dentin-derived growth factors. Thus, the regeneration of the pulp-dentin complex could be improved in response to microparticulate dentine in scaffold, which would provide both soluble and insoluble endogenous dentin proteins. Nevertheless, in vivo studies are necessary to elucidate the effect of soluble and insoluble dentin protein combination in regenerative endodontic therapy.

Release of total proteins showed that, except for Ch, all the scaffolds studied had released significant amounts of proteins from the 1st to the 32nd day. The addition of microparticulate dentin promoted a greater release of proteins, suggesting that a fraction of these proteins are from the dentin [40]. These findings are in accordance with Aubeux et al. [18], who observed that addition of dentin powder to the scaffold resulted in proteins release.

Besides the possibility to liberate growth factors, the morphology the scaffolds, like pores characteristics and superficial topography, is also of importance. In the present study, the scaffolds have pores ranging from 150 to 240 μm, which are the ideal size for penetration, adhesion and proliferation of cells [41,42]. Additionally, the addition of microparticulate dentin resulted in rough surfaces, and it is known that the increased of roughness surface improves cell adhesion and behavior [16].

Among the physicochemical characteristics, an adequate degradation allows the scaffold to be replaced by the newly-formed tissues [29]. In the present study, genipin was used as a cross-linking agent in order to limit the negative effect of rapid degradation of the scaffold made of chitosan and gelatin (data not shown). According to Kwon et al. [16], genipin has a higher biocompatibility than that of synthetic cross-linking agents, including ability to induce odontogenic differentiation of dental pulp stem cells. The biodegradation in group ChGDG was lower than in group Ch. On the other hand, cross-linked scaffolds had lower swelling rate compared to Ch scaffold. In this sense, one can infer that cross-linking limited the liquids absorption. Several authors have supported the use of hydrogels due to their adaptation to the root canal [43]. The swelling by ChGDG solid scaffolds would be favorable, since hygroscopic expansion might allow the scaffold to better adapt to the root canal. Therefore, in vivo studies are needed to assess whether the biodegradation and swelling rate reported here is adequate for endodontic regeneration.

Along with the physicochemical characteristics of the scaffold, their overall composition must be biocompatible. In the present study, the leachable substances of all scaffolds promoted cell viabilities higher than 60%. However, the medium conditioned by ChGDG scaffolds elevated the cell viabilities up to 100%. This finding could be resultant of induction by the proteins released from dentin.

Cell adhesion to the substrate is important to allow proliferation and differentiation of cells [44]. In the present study, no cell adhesion was observed in group Ch, a fact evidenced by the absence of spread cells on the surface of this scaffold, and this finding is in accordance to Kim et al. [8] and Asghari-Sana et al. [9]. This can be explained by the low hydrophilicity of chitosan [8] and the degree of deacetylation of chitosan [45,46]. It has been reported that low degree of deacetylation (<68%) of chitosan membranes limits the attachment and proliferation of cells [45], but a degree of deacetylation higher than 85% results in a great attachment and proliferation of cells [45,46]. In fact, the absence of adhesion in the present study could be due to the fact that a commercial chitosan was employed that presented the degree of deacetylation of 75–85%. Cell spreading was observed in groups containing proteins and cross-linking. The cross-linking of scaffolds may also have contributed to cell adhesion by improving their physicochemical characteristics and surface roughness [14,16]. In the ChGG it could be explained by the presence of gelatin, which is composed of a sequence of peptides involved in modulation of cell adhesion [9,15]. In ChGDG, cell spreading was improved by presence of gelatin and microparticulate dentin, which increased surface roughness and enhances cell adhesion.

Physiologically, all mammalian cells, except blood cells, are attached to a surface. Thus, proliferation will mostly occur in cells that were able to attach to the scaffold surface. This can explain why in the Ch group the proliferation of cells was smaller than those of the other groups, especially the ChGDG. Moreover, growth factors can be extracted from the dentin by the action of acetic acid used for dissolution of chitosan, which might explain the higher values found in scaffolds containing dentin. Corroborating our findings, Galler et al. [38] demonstrated that dentin proteins can promote proliferation of cells inoculated into the scaffold and favor new formation of tissue. Moreover, these authors highlighted that these proteins need to be immobilized in the scaffold. In this sense, chitosan is known to have the endogenous capacity to bind to growth factors [47,48]. Therefore, dentin proteins can be attached to chitosan during the scaffold preparation.

Clinically, the controlled apposition of hard tissue is desirable to outcome of regenerative endodontic treatment in immature teeth because it results in a uniform stress distribution and to increase the mechanical resistance of these teeth [49]. Thus for regenerative endodontics, the cells in the scaffolds must be able to differentiate in cells capable of producing mineralized extracellular matrix. One important assay to observe this capability is the Alizarin red, which allows the observation and measurement of such mineralized extracellular matrix. The Ch and the addition of gelatin and genepin to Ch somehow impaired the formation of mineralized matrix, however; the capability of the hCAPs to form such matrix was restored in the ChGDG group. Thus, one can infer that dentin was responsible for such finding. The osteoinductive effect of dentin, which occurs due to the presence of growth factors, is well known [19,50,51]. The addition of these factors to the scaffold and later release might explain the results. Genipin has also the capacity to promote formation of mineralized tissues [16], this could justify why in the ChGG the number of mineralized nodules were greater than in the plain Ch scaffold. Although such capability is of importance for regenerative endodontics, clinically the excess formation of such mineralization could be deleterious. If the reinvention is necessary, the complete obliteration of the root canal could complicate the endodontic retreatment.

Residual bacteria negatively impact the success of regenerative endodontic therapies [5]. Functionalized scaffold with antibacterial activity and suitable biological characteristics is a strategy little explored. The antibacterial activity of chitosan solutions has been reported elsewhere [52], but this effect is influenced by some factor such as pH, degree of deacetylation and the association with other chemicals [53]. In this sense, the greatest antibacterial activity of chitosan was reported at low pH [54] and high degree of deacetylation (e.g. > 75%) [55,56]. In addition, Sarasam et al. [57] observed that the antibacterial effect of chitosan is contact-dependent. In the present study, low molecular weight chitosan was used at 75–85% degree of deacetylation and neutralized pH by using multiple washes and lyonization process for obtaining solid scaffold. Therefore, the antimicrobial activity in group Ch within the first 6 h might be explained by the high rate of swelling in this experimental time, which may have resulted in a greater contact area between scaffold and bacterial suspension. Nevertheless, it was demonstrated that cross-linked chitosan has a lower antibacterial effect compared to pure chitosan, which might be related to a low rate of swelling. Low rates of degradation in the cross-linked scaffolds may have limited the availability of chitosan, thus meaning that the antibacterial effect may be contact-dependent. These results are in accordance to Sarasam et al. [57], Ducret et al. [13] and Radhika-Rajasree et al. [58] who reported bacterial grown in presence of chitosan-based scaffolds. In the 48-h, the ChGDG scaffold reduced the bacterial growth and limited the bacterial adhesion. The contact between leachable substances of ChGDG confirmed this antibacterial action from 24-h. This suggests that peptides can be released into the medium, which extends the antimicrobial activity. The result observed in groups Ch and ChGG might be explained by the degradation rate, and consequently, by the low availability of leachable substances of solid chitosan for inhibition of bacterial growth. In this sense, Smith et al. [20] observed that the organic fraction of the human dentin has antimicrobial peptides with bacteriostatic activity against *Streptococcus mutans*, *Streptococcus oralis* and *Enterococcus faecalis*. Additionally, Malmsten et al. [21] reported that that peptides derived from HB-EGF, amphiregulin, hepatocyte growth factor, PDGF-A and PDGF-B, and FGFs growth factors showed antimicrobial activity, causing membrane disruption of gram positive and negative bacteria, as well as fungi. This growth factors are present in the dentin matrix and could be extracted by acids [18–20,43,59] and ultrasonic activation [60]. Thus, the antimicrobial effects in ChGDG scaffold against E. faecalis might be explained by the extraction and immobilization of bioactive molecules from microparticulate dentin

during the scaffold elaboration process (e.g. ultrasonicated in acetic acid) and posterior release.

The characteristics of the ChGDG scaffold were those mostly expected of an ideal scaffold for regenerative endodontics. However, although the presence of dentin in this scaffold was pointed as the component that responded for the positive findings of the present study, clinically, the use of allogenic microparticulate dentin could be a limitation of the ChGDG scaffold. The immunogenic potential of collagen exposed in microparticulate dentin by acetic acid is not clear. Bakhtiar et al. [61] reported that atelopeptidization is necessary to decrease the immune reaction of EDTA treated dentin matrix xenograft. On the other hands, Chang et al. [62] demonstrated that EDTA treated sterilized allogenous dentin matrix promotes the stem cells differentiation without immunogenicity activity in in vivo assay. Nevertheless, in vivo studies are necessary to elucidate the immunogenic potential of the new scaffold developed in this study.

5. Conclusions

The new solid chitosan-based scaffold associated with gelatin, microparticulate dentin and genipin (ChGDG) has morphological, physicochemical, biological and antibacterial characteristics suitable for their potential use in regenerative endodontics. In this sense, the addition of microparticulate dentin improves the biological characteristics and promoted antibacterial activity of this scaffold that could act on residual bacteria in infected root canals besides controlling the stem cells differentiation for endodontic tissue regeneration.

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Appendix A. Preparation of microparticulate dentin

Nineteen crowns of human impacted third molars freshly extracted were used. The dental crown was separated from the root and the enamel was removed both using a diamond burs conical trunk in high rotation under refrigeration. The pulp tissue was removed from the pulp chamber using periodontal curettes, and the crowns were placed in sterile saline solution for a maximum period of 30 days at −80 °C. Next, the crowns were thawed and sectioned into 0.8 mm thick slices. Six grams of dentin slices were frozen in liquid nitrogen and crushed in an automatic ball mill for 6 cycles of 2 min each, at 30 Hz. The obtained powder was sieved using a 400-mesh sieve and stored at −80 °C.

The size distribution of the dentin particles was determined on the granulometer by laser diffraction (Helos) with a detection range of 0.1–350 μm: 0.15 g of the dentin powder was added at the 50 ml of distillated water and agitated at 1000 rpm for 1 min. The suspension was transferred to the analysis reservoir and ultrasonicated for 2 min, and then the evaluation started. This step was carried out on three samples in different mills in order to validate the reproducibility of the method.

The granulometric analysis demonstrated particles between 0.3 and 53 μm by showing a normal distribution characteristic curve (Appendix Fig. A1).

Appendix B. Isolation and immunophenotype Cell Characterization

Human immature impacted caries–free third molars with open apices (n = 4) were collected from the oral surgery clinics of the School of Dentistry, under local human research ethics committee approval (#3.612.573) and informed consent of the patients. Explants of the apical papilla are obtained from minced tissue fragments placed in clonogenic culture (alpha-MEM supplemented with 15% of bovine fetal serum, 100 μM of ascorbic acid, 2 mM of L-glutamine, 100 U/mL of penicillin and 100 μl of streptomycin). The cells were trypsinized and plated

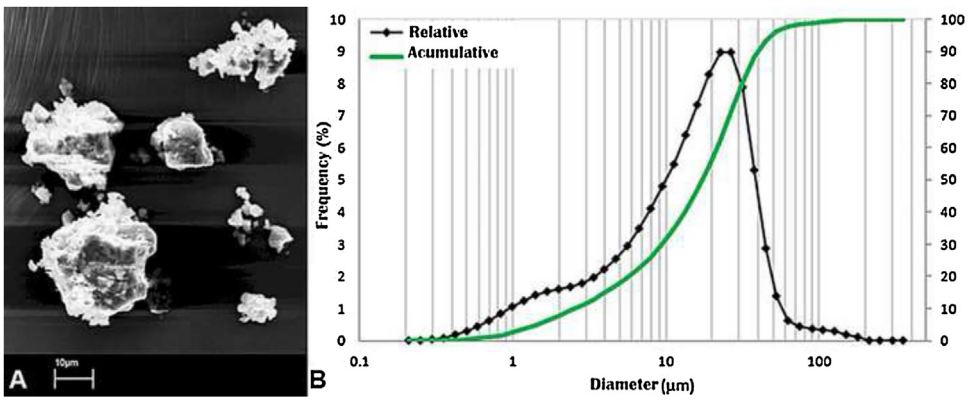

Fig. A1 – (A) Electronmicrograph and (B) granulometric distribution of microparticulate dentin.

after reaching subconfluence. This procedure was repeated until the cells were at the third passage. The cells (1 × 106 cells/per antibody) were washed and resuspended in PBS containing saturating concentrations of MSC-associated markers (CD105, STRO-1 and CD146) and non-MSC-associated markers (CD14) (Santa Cruz Biotechnology). The cells were sorted in a flow cytometer (BD Biosciences) and a total of 50,000 events were analyzed by using the FlowJo Software (Tree Star)

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