BIOLOGICAL AND BACTERICIDAL PROPERTIES OF AG-DOPED BIOACTIVE GLASS IN A NATURAL EXTRACELLULAR MATRIX HYDROGEL WITH POTENTIAL APPLICATION IN DENTISTRY

Y-Y. Wang[1,2,*], X. Chatzistavrou[1,*], D. Faulk[3], S. Badylak[3,4,5], L. Zheng[1], S. Papagerakis[6,7], L. Ge[2], H. Liu[2,§] and P. Papagerakis[1,8,9,§,*]

[a] Department of Orthodontics and Pediatric Dentistry, School of Dentistry, University of Michigan, Ann Arbor, MI, USA
[b] Department of Pediatric Dentistry, School & Hospital of Stomatology, Peking University, Beijing, China
[c] McGowan Institute for Regenerative Medicine, University of Pittsburgh
[d] Department of Surgery, University of Pittsburgh
[e] Department of Bioengineering, University of Pittsburgh
[f] Department of Otolaryngology, Medical School, University of Michigan, Ann Arbor, MI, USA
[g] Department of Periodontics & Oral Medicine, School of Dentistry, University of Michigan, Ann Arbor, MI, USA
[h] Center for Organogenesis, Medical School, University of Michigan, Ann Arbor, MI, USA
[i] Center for Computational Medicine & Bioinformatics, Medical School, University of Michigan, Ann Arbor, MI, USA

[*] These authors contribute equally as first authors.
[§] Co-equal senior authors.

Abstract

The aim of this study was the fabrication and evaluation of a novel bioactive and bactericidal material, which could have applications in dentistry by supporting tissue regeneration and killing oral bacteria. Our hypothesis was that a new scaffold for pulp-dentin tissue engineering with enhanced antibacterial activity could be obtained by associating extracellular matrix derived from porcine bladder with an antibacterial bioactive glass. Our study combines *in vitro* approaches and ectopic implantation in scid mice. The novel material was fabricated by incorporating a sol-gel derived silver (Ag)-doped bioactive glass (BG) in a natural extracellular matrix (ECM) hydrogel in ratio 1:1 in weight % (Ag-BG/ECM). The biological properties of the Ag-BG/ECM were evaluated in culture with dental pulp stem cells (DPSCs). In particular, cell proliferation, cell apoptosis, stem cells markers profile, and cell differentiation potential were studied. Furthermore, the antibacterial activity against *Streptococcus mutans* and *Lactobacillus casei* was measured. Moreover, the capability of the material to enhance pulp/dentin regeneration *in vivo* was also evaluated. Our data show that Ag-BG/ECM significantly enhances DPSCs' proliferation, it does not affect cell morphology and stem cells markers profile, protects cells from apoptosis, and enhances *in vitro* cell differentiation and mineralisation potential as well as *in vivo* dentin formation. Furthermore, Ag-BG/ECM strongly inhibits *S. mutans* and *L. casei* growth suggesting that the new material has also anti-bacterial properties. This study provides foundation for future clinical applications in dentistry. It could potentially advance the currently available options of dental regenerative materials.

Keywords: Ag-doped bioactive glass, natural extracellular matrix hydrogel, dental pulp stem cells, biological properties, antibacterial activity.

*Address for correspondence:
Petros Papagerakis, DDS, MS, PhD
School of Dentistry, 1011 North University
Ann Arbor, MI, 48109, USA
Telephone number: +1 210 845 9190
E-mail: petrosp@umich.edu

Introduction

It has been observed that two-thirds of all restorative dentistry involves the replacement of failed restorations (Maupomé and Sheiham, 1998; Burke *et al*., 1999). The development of innovative bactericidal, bioactive/biomimetic ion-releasing restorative materials that could heal and regenerate the defect area, consists one of the main targets in dental biomaterials research (Sauro *et al*., 2013).

Regarding pulp-dentin regeneration, several types of scaffolds have been tested combined with dental pulp stem cells (DPSCs) both *in vitro* and *in vivo* (Demarco *et al*., 2010; Wang *et al*., 2010; Galler *et al*., 2012). However, all those studies regenerated only a limited number of dental tissues, while none of them has been used in clinical trials. The challenge to develop a suitable scaffold that can provide a suitable microenvironment for DPSC proliferation, differentiation and new tissue formation still remains.

Towards this aim, natural extracellular matrix (ECM) scaffolds are considered advantageous because of their inherent bioactive properties and ability to mimic the native microenvironment niche (Currie *et al*., 2001; Patino *et al*., 2002). ECM hydrogels have been successfully isolated and used in several regenerative medicine applications (Turner *et al*., 2010; Valentin *et al*., 2010; Singelyn *et al*., 2012). ECMs have been shown to promote tissue healing and regeneration in a number of anatomic locations, including skeletal muscle (Marelli *et al*., 2010; Mase *et al*., 2010), oesophagus (Nieponice *et al*., 2009) and urinary bladder (Boruch *et al*., 2010). However, it is not clear if they can inhibit pathogenic bacterial growth and prevent secondary caries formation (Ljungh *et al*., 1996). Moreover, natural ECM scaffolds have not been tested in detail for dental applications.

Bioactive glasses (BGs) are considered to be osteoconductive and osteoinductive, regulating genetic profiles of osteoprogenitor/stem cells down to the lineage of bone forming cells (Rezwan *et al*., 2006). Bioactive glasses are extensively used for bone reconstitution and tissue engineering (Blaker *et al*., 2003; Cushing *et al*., 2004), while they are also significant candidates for the remineralisation of tooth tissues (Forsback *et al*., 2004). However, BGs have not been shown to promote stem cell migration, as do ECM scaffolds. Moreover, BGs delivery needs to be facilitated by appropriate scaffold matrices.

Therefore, the idea of using natural extracellular matrix hydrogels (ECMs) in concert with bioactive glasses is considered to be a promising strategy to attain cell matrices with physic-chemical and biological properties appropriate for tissue engineering.

On the other hand, several antimicrobial agents have been incorporated into dental restorative materials and bonding systems (Imazato *et al*., 2003) to prevent secondary caries. However, there is no definitive evidence that these antibacterial agents are effective in improving clinical outcomes (Pereira-Cenci *et al*., 2009). The antimicrobial use of silver (Ag) compounds tends to be restricted to the extensive application of silver nitrate, silver foil, and silver sutures for the prevention and treatment of ocular, surgical, and dental infections. It has been observed that silver interacts with sulphydryl groups of proteins and with DNA, altering hydrogen bonding and inhibiting respiratory processes, DNA unwinding, cell-wall synthesis, and cell division (Oppermann *et al*., 1980; Lansdown, 2002; 2006). These interactions critically effect bacterial killing and inhibit biofilm formation at the macro level (Wu *et al*., 2007). The interaction of silver with thiol groups is considered to be the central mechanism of these diverse effects (Russell *et al*., 1994).

We have recently fabricated a new sol-gel derived silver-doped silicate glass (Ag-BG) that presents strong anti-bacterial and bioactive properties (Chatzistavrou *et al.*, 2014). Ag-BG can effectively inhibit bacteria growth, by the ion release process of Ag. Moreover, the composition of Ag-BG presents Ca/P ratio around 4 which can enhance the deposition of apatite phase (Chatzistavrou *et al*., 2012). This remineralisation process can be described as a slow dissolution/precipitation process of mineral constituents into the dentin matrix. Apatite could subsequently develop a strong bond with the surrounding tooth tissue, eliminating the possibility of future micro-penetrations. Similar compositions have been already used for bone repair applications, both at the experimental and clinical levels (Kellomaki *et al.*, 2000). In dentistry, silver containing bioactive glass with different composition has been also reported (Balamurugan *et al*., 2008); however, the proposed injectable combination of Ag-BG with ECM has not been adopted before.

Many studies have also revealed that silicate bioactive glasses stimulate the growth and osteogenic differentiation of human primary osteoblasts (Xynos *et al*., 2000; Hench *et al*., 2004; Rahaman *et al*., 2011). However, to date, the effect of a silver doped silicate bioactive glass on human DPSCs is unclear. We hypothesise that the release of soluble silicon ions (Si^{4+}) from the degradation of Ag-BG will contribute leading to favourable intracellular and extracellular responses promoting odontogenic differentiation of DPSCs.

The aim of this work is to generate and characterise a novel complex system (Ag-BG/ECM), fabricated through the incorporation of Ag-BG into natural ECM hydrogel. Our research hypothesis is that ECM facilitates the delivery of Ag-BG, making it injectable and potentially provides niche-like signals to DPSC. This work suggests that Ag-BG/ECM is able to stimulate and accelerate human DPSCs growth, differentiation potential and enhanced dentin formation *in vivo*, while also providing strong bactericidal properties against *S. mutans* and *L. casei*.

Materials and Methods

Sample preparation

Ag-BG

The fabrication of the Ag-BG in the systems SiO_2 58.6-CaO 24.9-P_2O_5 7.2-Al_2O_3 4.2-Na_2O 1.5-K_2O 1.5-Ag_2O 2.1 wt. %, has been already described (Chatzistavrou *et al*., 2012). Briefly, the fabrication protocol is based on the sol-gel process by mixing the solution stage of the 58S sol-gel bioactive glass (in the system SiO_2 58-CaO 33-P_2O_5 9 wt.%), with the respective solution stage of the sol-gel glass in the system SiO_2 60-CaO 6-P_2O_5 3-Al_2O_3 14-Na_2O 5-K_2O 5- Ag_2O 7 wt.%, as it has been presented in detail in our previous work (Chatzistavrou *et al*., 2010; Chatzistavrou *et al*., 2012). After extended stirring, the final homogeneous solution follows a specific heat treatment: aging at 60 °C, drying at 180 °C and stabilisation up to 700 °C. The fabricated material was in powder form with particle size < 35μm.

ECM

The ECM hydrogels were provided by University of Pittsburgh. They were prepared from decellularised porcine urinary bladder matrix (UBM), as previously described (Freytes *et al*., 2004). Briefly, porcine urinary bladders from market weight pigs were harvested, and the urothelial, serosal and muscular layers were removed by mechanical delamination. The remaining tissue consisted of intact basement membrane and tunica propria, which was rinsed with deionised water and then treated with 0.1 % peracetic acid/4 % ethanol on an orbital shaker at 300 rpm for 2 h. The UBM was then rinsed twice with phosphate buffered saline (PBS) for 15 min each followed by two rinses of 15 min in deionised water. UBM samples were frozen and lyophilised for use in hydrogel preparation (Freytes *et al*., 2008; Hong *et al.*, 2011). In brief, lyophilised ECM scaffolds were powdered using a Wiley Mill and filtered through a 40-mesh screen. The comminuted ECM was then enzymatically digested in a solution of 1 mg/mL porcine pepsin (Sigma Aldrich, St. Louis, MO) in 0.01 N HCl under a constant stir rate for 72 h at room temperature. ECM pepsin digest stock solutions of 10 mg ECM/mL (dry weight) were frozen until use in subsequent experiments. Gelation was induced by neutralising the pH and salt concentration of the pepsin digest at 4 °C followed by warming to 37 °C. Neutralisation was accomplished by the addition of one-tenth the digest volume of 0.1 N NaOH, one-ninth the digest volume of 10× PBS, and then diluting to the desired final ECM concentration with 1× PBS while on ice. The neutralised digest (pre-gel) was then placed in a non-humidified incubator heated to 37 °C for 1 h, after which, a hydrogel had formed. ECM concentration of 10 mg/mL was prepared.

Ag-BG/ECM

Pre-gel was mixed by pipetting with Ag-BG powder. The Ag-BG was incorporated into ECM at ratio 1:1 in wt.%

(Ag-BG/ECM). The Ag-BG/ECM was observed injectable through a syringe and 27G needle to subjectively determine injectability.

The extracts of Ag-BG/ECM and ECM alone were collected by incubating them into cell culture medium at equal volumes for 24 h at 37 °C. The extracts were collected after centrifuging. Undiluted extracts of all tested materials (1 mL for each material) were serially diluted (to 1:2,1:4 and 1:8 – v:v) for the assessment of potential dose-response effects. Cell culture medium free of materials' extracts and incubated under the same conditions was used as negative control at all cell culture studies (UNTREATED). The biological response of the DPSCs on the prepared dilutions and the extracts-free medium was studied.

Cell culture-Determination of cell viability, proliferation and cell apoptosis

Viability

The laboratory of Dr. Kaigler at the University of Michigan kindly provided DPSCs. DPSCs were isolated applying that pulp tissue was gently separated from the crown and root, and subsequently digested in a solution of 3 mg/mL collagenase type I (Sigma, St. Louis, MO, USA) and 4 mg/mL dispase (Sigma, St. Louis, MO, USA) for 1 h at 37 °C. Single-cell suspensions were obtained by passing the cells through a 70-mm strainer (Falcon). Single-cell suspensions (0.5 to 1.0 × 10^3/well) were seeded onto 6-well plates (Costar) containing alpha modification of Eagle's medium (GIBCO/BRL) supplemented with 15 % foetal bovine serum (FBS; Hyclone), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma, St. Louis, MO, USA), and incubated at 37 °C in 5 % CO_2. The DPSCs were confirmed to be actually stem cells according to the methods described previously. DPSCs were then cultured for 24 h at 37 °C on 96-well plates with density 5 × 10^3 cells/well. They were cultured with the different dilutions of the extracts and the extract-free medium (UNTREATED). The cell viability was evaluated using the cell counting kit-8 (CCK-8) assay and repeated five times for each sample. The dilution of the extracts of the samples, at which DPSCs showed the highest viability, was used at all the experiments presented in this work.

Proliferation

The proliferation of the DPSCs was evaluated. DPSCs were in culture with the extracts of the samples at the specific dilution with the highest cell viability and compared with the UNTREATED. DPSCs (1.0 × 10^3cells/well) expanded *ex vivo* and seeded onto five 96-well plates separately. After 1, 3, 5 and 7 days of culture, CCK-8 assay was used and repeated four times to evaluate the cell proliferation. During the culture, the morphology of DPSCs in each group was recorded by Nikon optical microscopy.

Cell apoptosis: Flow cytometry

Cell apoptosis rate was observed by flow cytometry. DPSCs cultured for 24 h at 37 °C with the extracts of Ag-BG/ECM and ECM alone at the dilution with the highest cell viability and also untreated cells as negative control were stained with FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) according to the manufacturer's instructions. Stained cells were analysed by the Beckman Coulter MoFlo® Astrios™ flow cytometry system. Viable cells were negative for both PI and annexin V-FITC; apoptotic cells were positive for annexin V-FITC and negative for PI, whereas late apoptotic dead cells displayed strong annexin V-FITC and PI labelling. Non-viable cells, which underwent necrosis, were positive for PI but negative for annexin V-FITC. The cell apoptosis experiments were repeated three times.

Effects of Ag-BG/ECM and ECM on stem cells markers profile

Immunofluorescence staining

DPSCs were subcultured on 12-chamber slides (2.0 × 10^4/well, second passage) and grown to 80 % confluence. The cells were then incubated for 24 h at 37 °C, with the extracts of the materials at the dilution with the highest viability as well as the extract-free medium. Standard immunofluorescence methods were performed to detect potential changes in stem cell markers profiles. Briefly, DPSCs were initially fixed in 4 % paraformaldehyde and the no-specific antigens were blocked with PBS containing 10 % bovine serum albumin at room temperature for 1 h. The cells were then incubated with diluted (1:50) primary antibody CD73 (Biolegend), CD90 (BD Pharmingen) and CD105 (Biolegend) overnight at 4 °C, washed with PBS and subsequently incubated with fluorescein-conjugated secondary goat anti-mouse polyclonal antibody (Invitrogen) at room temperature in the dark for 1 h. DAPI (Invitrogen) staining was then performed in the dark for 5 min. After being washed with PBS, the slides were analysed using a fluorescence microscope (Olympus BX-51, Tokyo, Japan).

Flow cytometric study

In order to evaluate potential changes in the mesenchymal phenotype of the DPSCs after being exposed to the Ag-BG/ECM and ECM diluted extracts, the surface antigens of DPSCs were analysed by flow cytometry. Cells were trypsinised and incubated in PBS for 60 min with fluorescein conjugated antibodies against CD73 (Brilliant Violet421, Biolegend), CD90 (FITC, BD Pharmingen), CD105 (PE, Biolegend). The flow cytometry analysis of cells was carried out using Beckman Coulter MoFlo® Astrios™ flow cytometry system, with 500,000 events being counted for each case. The procedure was repeated three times.

DPSCs odontogenic differentiation

Previous studies of solid polymer scaffolds have shown that differentiation of DPSC may be stimulated by the presence of dentine (Sakai *et al*., 2011). In our study, we used sterilised pulpless tooth slice incubated in culture medium to stimulate the odontogenic differentiation. DPSCs in 1 × 10^5 cells were centrifuged and the fabricated pellet was mixed with Ag-BG/ECM and ECM separately. Then, each of the complex mixture of cells with material was seeded in the hole of the tooth slice. Cells alone were seeded in tooth slice and cells only without the present of tooth slice were also seeded on the well plate. These two groups were designed as controls. Regular and

Table 1: List of the DNA sequences of the primers used for the RT-PCR analysis.

Gene name	5'-squence-3'	Product size (bp)	GenBank number
GAPDH	Forward: GCAAATTCCATGGCACCGTC	819	XM_003273723.2
	Reverse: GGTCCACCACCCTGTTGCTA		
DMP-1	Forward: CTGTGCTCTCCCAGTAACCA	159	NM_004407.3
	Reverse: CTGGGGTCTTCATTTGCCTG		
RUNX2	Forward: TCCCCGTCCATCCACTCTAC	157	NM_001024630.3
	Reverse: CACCTGCCTGGCTCTTCTTA		
ALP	Forward: GCGCAGGACAGGATTAAAGC	246	NM_014476.5
	Reverse: TCCACTGCCACAGTCAATCC		
DSPP	Forward: CATGGGCCATTCCAGTTCCTC	152	NM_014208.3
	Reverse: TTCATGCACCAGGACACCAC		

differentiation cell culture media were used for culture up to 21 days. The differentiation was measured applying RT-PCR and Alizarin Red staining, following the procedure that has been reported elsewhere (Gronthos *et al*., 2000). The intensity of the staining was quantified by using image J. The DPSCs odontogenic differentiation study was repeated three times.

RT-PCR

Total RNA was isolated from cells after 21 days using TRIzol (Invitrogen, Carlsbad, CA), and 2 μg of RNA was reverse transcribed with TaqMan reverse transcription reagents (Applied Biosystems, Branchbury, NJ), following the manufacturer's instructions. The resulting cDNA was then amplified by RT-PCR using AmpliTaq Gold DNA Polymerase (Applied Biosystems). RT-PCR amplifications were performed at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s using specific primers for GAPDH, DMP-1, Runx2, ALP and DSPP. The design of the primers was based on published mouse cDNA sequences (Table 1).The RT-PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI) and RNA expression was confirmed by sequencing.

Antibacterial activity

Equal volumes of Ag-BG/ECM and PBS, which was used as control, were mixed separately with *S. mutans* (ATCC 25175) and *L. casei* (ATCC 15008) (kindly provided by Dr. J.C. Fenno at the University of Michigan) and then incubated for 24 h at 37 °C. After 24 h, each mixture was diluted serially (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) and subsequently was plated on agar plates. All plates were incubated for 48 h at 37 °C, and the developed colonies were counted.

Pulp/dentin *in vivo* formation

An approved UCUCA protocol for small animals was applied during this study. In particular, approximately 1.0×10^7 DPSCs cells (third passage) were cultured with Ag-BG/ECM extracts in 1/4 dilution and, as a control, the same density of DPSCs cells were cultured with extract-free medium. Both groups of DPSCs cells were then co-cultured separately with 40 mg of hydroxyapatite (HA) powder for 3 h, before they were implanted into the dorsal surfaces of 10-week-old mice with severe combined immunodeficiency (CB-17 scid mice, NIH-bg-nu-xid; Harlan-Sprague-Dawley; Harlan Laboratories, Indianapolis, IN). Six scid mice were used for the experiment. Each nude mouse received both experimental and control group cells/materials in opposite sites. Thus, we totally analysed six different samples for the experimental group and six different samples for the control group. The implants were recovered after 8 weeks, then fixed with 4 % formalin, decalcified with buffered 10 % EDTA (pH 8.0), and finally they were embedded in paraffin. Sections (5 mm) were deparaffinised and stained with haematoxylin-eosin. Dentin formation was quantified using image J (NIH Image software).

The immunohistochemical staining was carried out according to the manufacturer's instructions of VECTASTAIN ABC system, 3,3' Diaminobenzidine (DAB) was applied as the chromogen. Primary antibodies used are: DSP (gift of Dr. Simmer at the University of Michigan; dilution 1:500), nestin (Novus USA; dilution 1:200), VEGF (Chemicon Millipore; dilution 1:200) and Anti-nuclei antibody, clone 235-1 (Chemicon Millipore; dilution 1:500).

Statistical analysis

SPSS software was used (version 13.0; SPSS, Chicago, IL, USA). Statistical analysis was performed using the paired Student's *t* test. The level of statistical significance was $p < 0.05$.

Results

Cell viability, proliferation and cell apoptosis

The cell viability of DPSCs in culture with different dilutions of the extracts of the Ag-BG/ECM and ECM alone was evaluated. It was found that cell viability was slightly inhibited for undiluted extracts of both materials. However, this inhibition was not significant compared to the controls. In contrast, there was increase in cell viability for both materials at 1:2, 1:4 and 1:8 dilutions. However, the cell viability is statistically significant compared to the control only for Ag-BG/ECM at dilutions 1:4 and 1:8 ($p < 0.005$). The 1:4 dilution for both materials showed

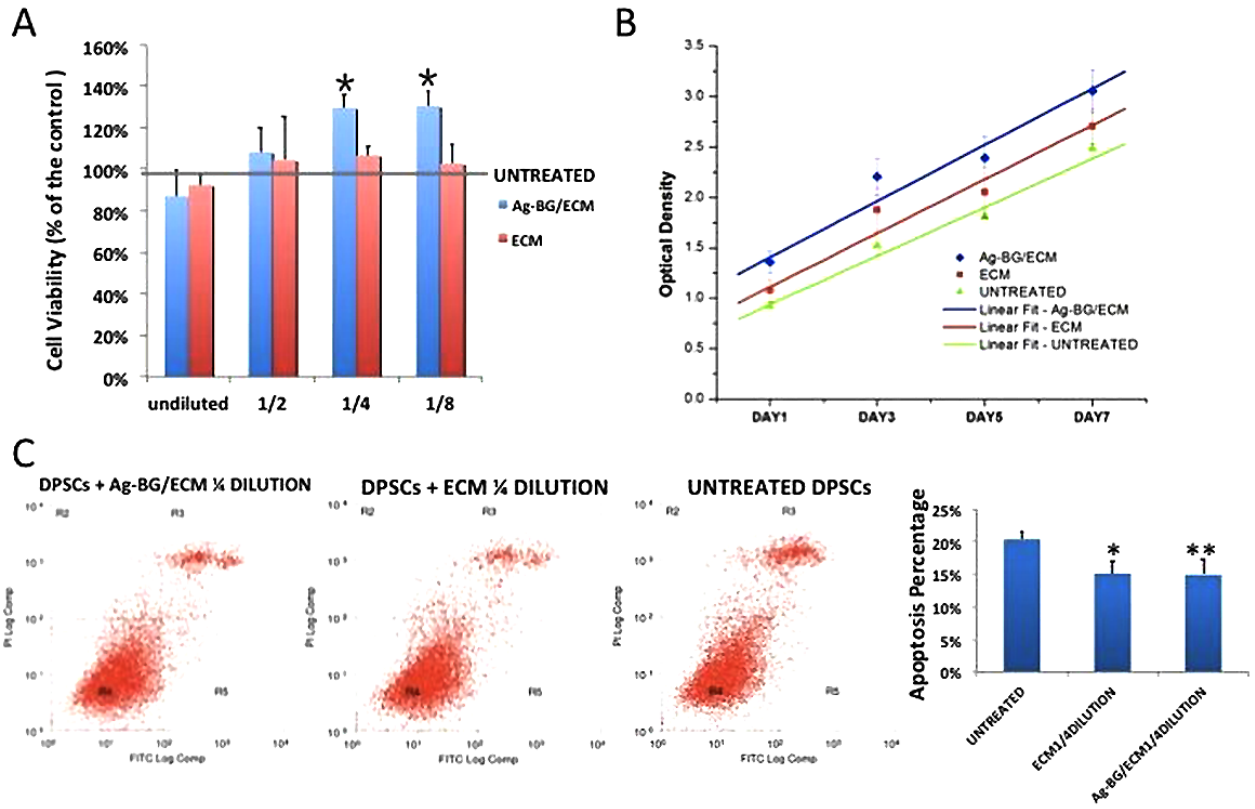

Fig. 1. (**A**) The viability of DPSCs as percentage of live cells of the extract-free control was measured after 24 h of culture and compared with the extracts of Ag-BG/ECM and ECM alone at different dilutions (* = statistically significant difference between control and Ag-BG/ECM group ($p < 0.05$)). (**B**) Proliferation of DPSCs after 1, 3, 5 and 7 days in culture with Ag-BG/ECM and ECM extracts at 1:4 dilution and control. (**C**) Statistical results of apoptosis rate derived from flow cytometry study showed that DPSCs treated with 1:4 diluted extracts of Ag-BG/ECM and ECM have significant lower apoptotic rate than the control group (* = statistical significant difference between control and ECM; ** = statistical significant difference between control and Ag-BG/ECM ($p < 0.05$)).

slightly higher cell viability compared to 1:8 dilution and it was finally chosen as the working dilution of both materials in the rest of our studies (Fig. 1A).

Cell proliferation showed a linear increase from day one to day seven of culture for both materials and the control group (cells without treatment). The proliferation rate was calculated similarly in all cases. However, at each time point the cell proliferation in the Ag-BG/ECM samples was higher compared to the respective ECM samples. ECM samples showed higher cell proliferation compared to controls (Fig. 1B).

Apoptosis rate of DPSCs was found to be significantly lower in case of culture with the diluted extracts of Ag-BG/ECM and ECM ($p < 0.05$) compared to the control group (cells without treatment) (Fig. 1C), while there was no significant difference in case of Ag-BG/ECM and ECM alone ($p > 0.05$).

Stem cells markers profile

The DPSCs expressed MSCs markers CD73, CD90 and CD105 when they were cultured for 24 h with the extracts of the Ag-BG/ECM and ECM diluted at 1:4, as presented by immunofluorescence staining (Fig. 2A). Their phenotypical characteristics remain unchanged compared to the control group (cells without treatment). Positive expression of the MSCs markers CD73, CD90 and CD105 was also found by flow cytometry for all samples (Fig. 2B). There is no significant difference in the positive expression percentage of MSCs markers among the three different groups (Ag-BG/ECM, ECM and control) (Fig. 2B).

DPSCs odontogenic differentiation

The DPSCs showed odontogenic differentiation after 21 days in culture in the presence of a tooth slice alone or in the presence of a tooth slice with Ag-BG/ECM and ECM. However, the calcium deposition and gene expression varied significantly among the different groups (Fig. 3). No signs of differentiation were observed at the DPSCs without the presence of a tooth slice. The tooth slice with Ag-BG/ECM/DPSCs showed significantly higher calcium deposition compared to the tooth slice with ECM/DPSCs and to the tooth slice/DPSCs alone (Fig. 3A). The odontogenic differentiation markers DMP1, Runx2 and ALP are up-regulated significantly in the tooth slice with Ag-BG/ECM/DPSCs compared to other groups, but no DSPP is detected (Fig. 3B). The addition of differentiation medium (incorporating Ascorbic acid, Dex and β-Glycerophosphate) and culture for 10 days enhanced

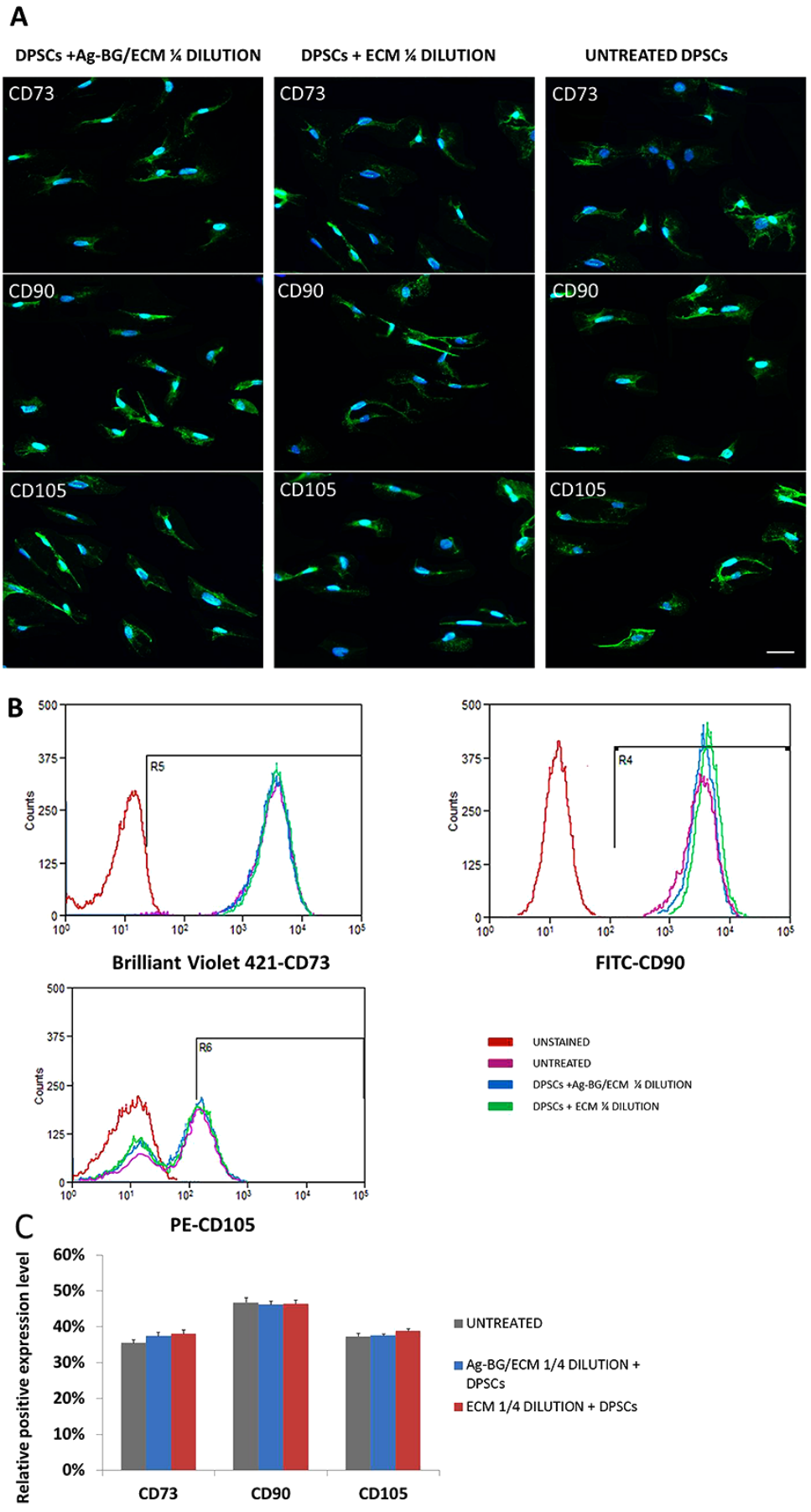

Fig. 2. (**A**) Immunofluorescence staining applied to observe the expression of the MSCs markers CD73, CD90 and CD105 in DPSC (bar = 200μm). (**B**) Flow cytometry study showed the positive expression of MSCs markers CD73, CD90 and CD105 in DPSC (**C**) Statistical analysis of the flow cytometry results.

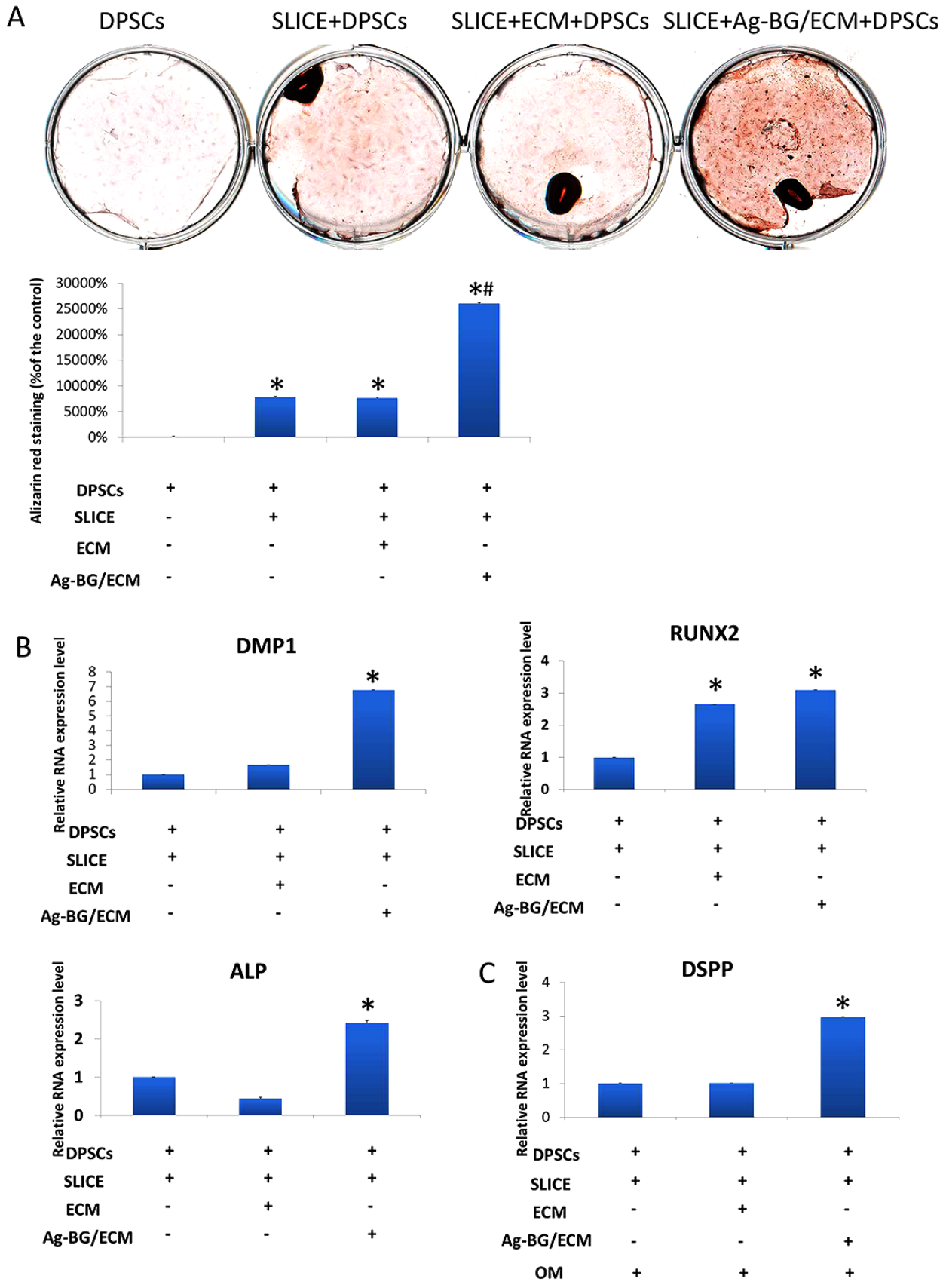

Fig. 3. (**A**) Odontogenic differentiation by alizarin red staining (* = statistical significant difference between the DPSCs alone and the other three groups) (# = statistical significant difference between the Ag-BG/ECM and the other three groups) ($p < 0.05$). (**B**) RT-PCT analysis showed the expression levels of the odontogenic related markers (DMP-1, RUNX2 and ALP) (* = statistical significant difference compared to the control) ($p < 0.05$). (**C**) RT-PCR results on DSPP expression after culture for 10 more days with mineralisation medium (* = statistical significant difference compared to the tooth slice/DPSCs group) ($p < 0.05$).

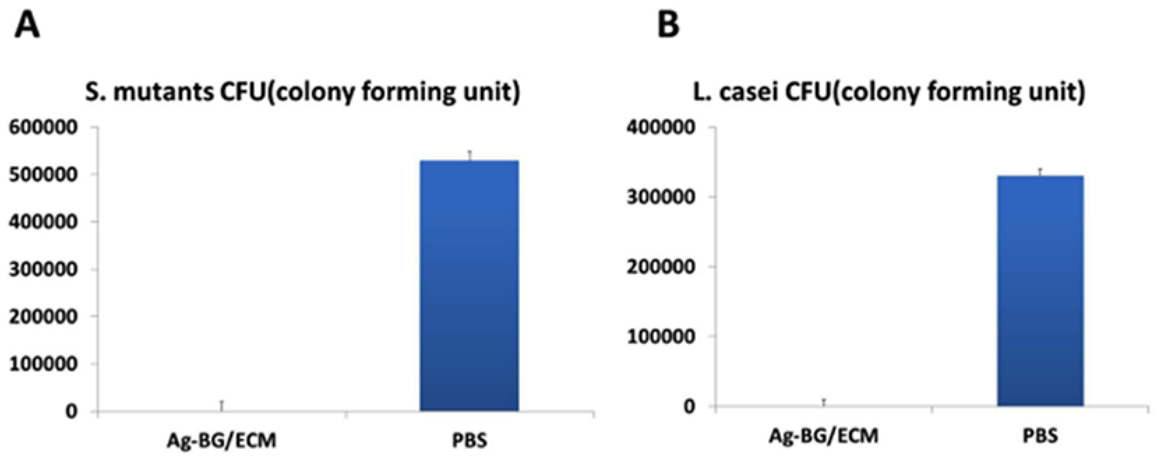

Fig. 4. Antibacterial activity of Ag-BG/ECM against *S. mutans* (**A**) and *L.casei* (**B**). No colonies growth is found in the presence of Ag-BG/ECM.

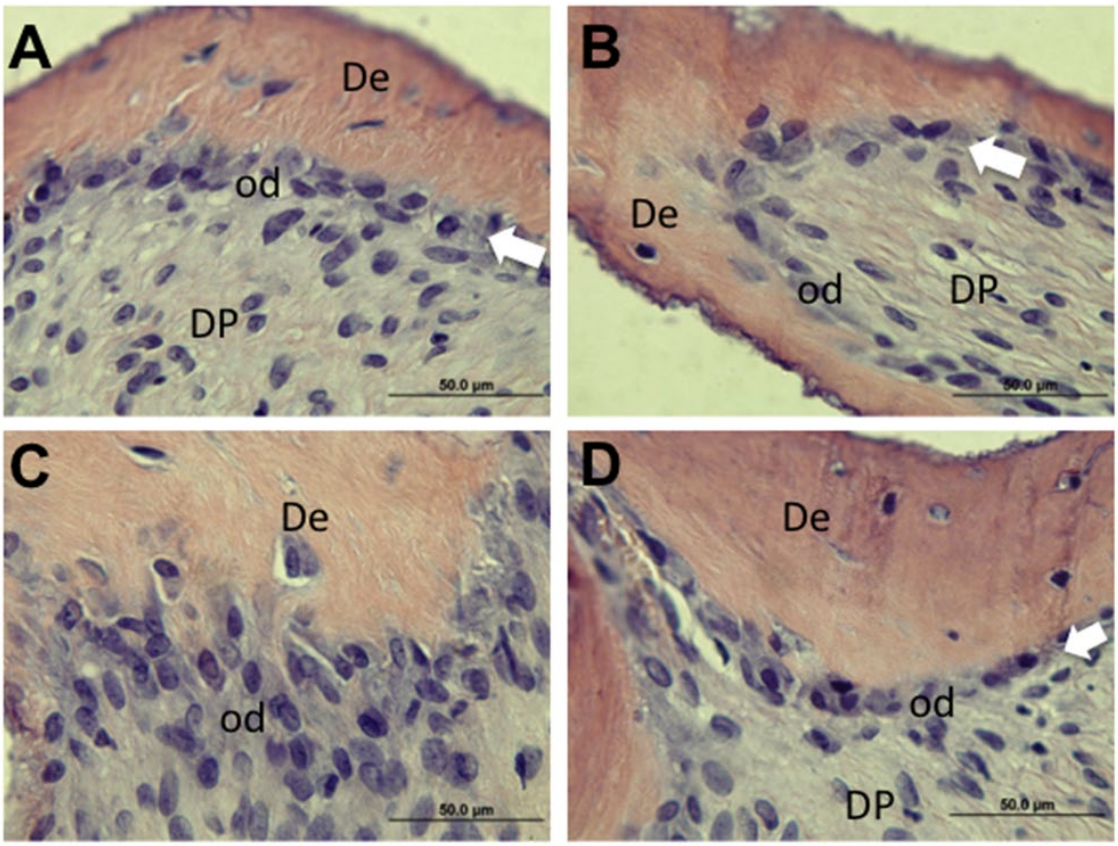

Fig. 5. (**A-D**) The regenerated mineralised tissue shows a dentin-like (De) morphology with the collagenous matrix being deposited perpendicular to the odontoblast-like (od) layer. The odontoblast-like cells lining the regenerated dentin-like tissue (white arrows) present characteristic morphological features of odontoblasts. The new dentin/pulp like complex includes also cells with fibroblastic morphology similar to dental pulp (DP) cells underlying the odontoblast-like cells. All four images were taken at 100× magnification.

the expression of DSPP. Tooth slice with Ag-BG/ECM/DPSCs showed significant increase of DSPP expression compared to the other groups (Fig. 3C).

3.4 Antibacterial activity

The antibacterial activity of Ag-BG/ECM against *S. mutans* and *L.casei* was evaluated (Fig. 4A-B). Significantly, there is no bacteria growth after 24 h of co-culture. This activity is significant different compared to the control (PBS co-culture with bacteria).

Pulp/dentin *in vivo* formation

Both groups (the DPSCs cultured with Ag-BG/ECM extracts and mixed with hydroxyapatite (HA) scaffold constitute the experimental group; the DPSCs cultured with extract-free medium and HA constitute the control group) after eight weeks of implantation induce the formation of pulp/dentin complex. The formed tissues show dentin-like morphology with the collagenous matrix being deposited perpendicular to the odontoblast-like layer when they are observed on the surface of the hydroxyapatite particles

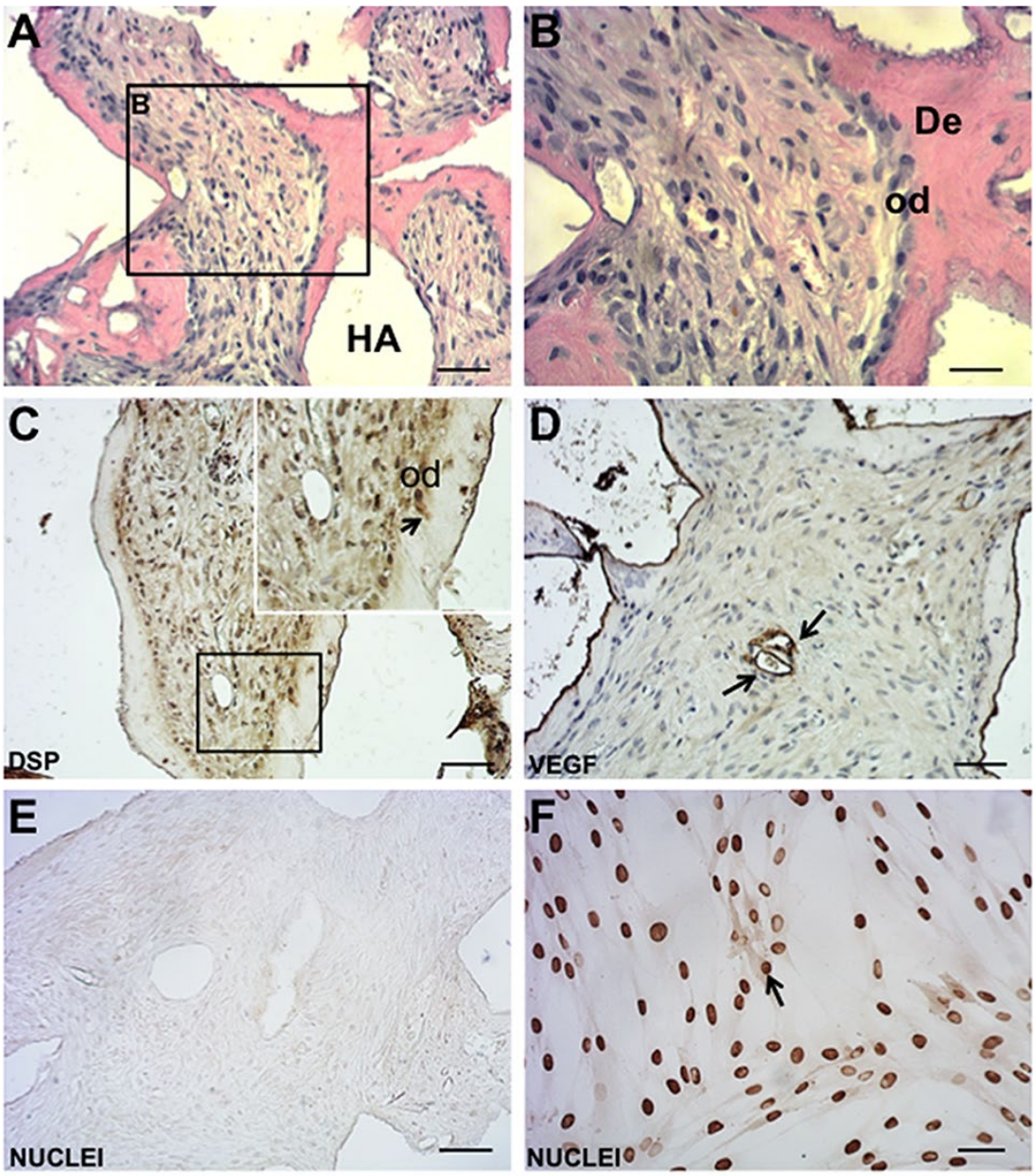

Fig. 6. (**A**, **B**) New dentin/pulp like complex was formed by both groups used (the DPSCs cultured with Ag-BG/ECM extracts and HA constitute the experimental group; the DPSCs cultured with extract-free medium and HA constitute the control group). The HA carrier surfaces were lined with a dentin-like matrix (De), and an interface layer of odontoblast-like (od) cells. A magnified view of the dentin matrix (De) highlights the odontoblast-like layer (od) and odontoblast processes (**B**). Abbreviations: HA, hydroxyapatite; (**C**, **D**) New dentin/pulp like complex showed positive staining for DSP (**C**) and VEGF (**D**); Higher magnification of the dentin-like regenerated tissue reveals positive signal in odontoblast-like cells processes (arrow); (**E**, **F**) New dentin/pulp like complex showed negative staining for the anti-nuclei human antibody eight weeks after implantation (**E**). Immunohistochemistry with the anti-nuclei human antibody on the human DPSCs was used as a positive control for this experiment (**F**) (Bar = 200 μm in **A**-**E**, 20 μm in **F**).

(Fig. 5 and Fig. 6A-B). The dental mineralising cells facing dentin-like matrix display protruding cytoplasmic processes into the matrix and are positively immunostained for DSP (Fig. 6C), the main phenotypic marker of odontoblasts. This dentin-like matrix is interfaced with a pulp-like interstitial tissue that includes blood vessels (VEGF positive) (Fig. 6D). The new-formed dentin/pulp-like complex shows negative response on the anti-human nuclei antibody staining at eight weeks after implantation (Fig. 6E) compared to anti-human nuclei antibody positive control staining (Fig. 6F). Of relevance, a significant higher number of odontoblast-like cells immunopositive for DSP is observed for the Ag-BG/ECM treated group compared to control group (Fig. 7A-B). Moreover, the Ag-BG/ECM treated group induces significantly higher dentin formation compared to control group (Fig. 7C-E), which is the

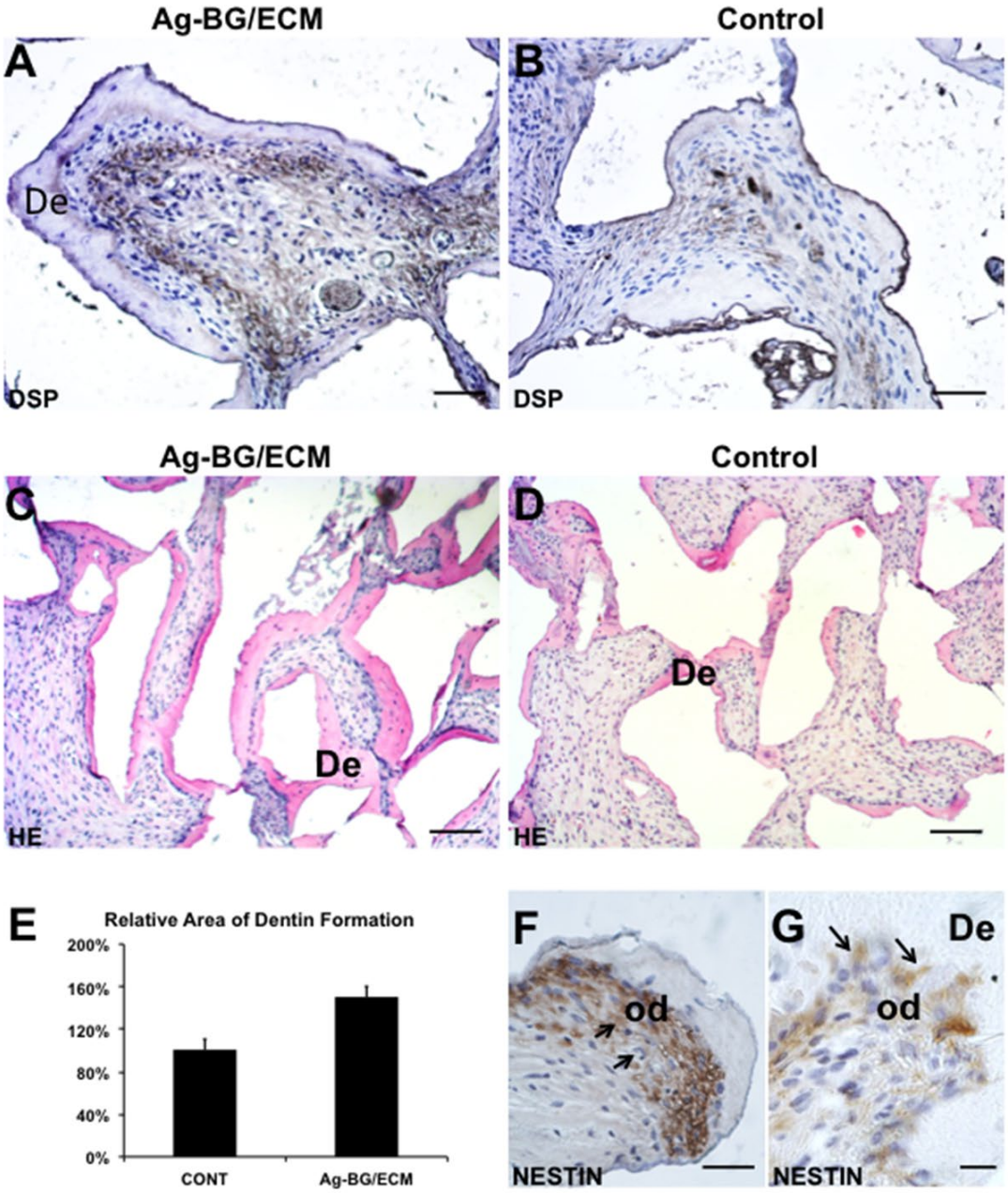

Fig. 7. (**A-E**) Comparison of dentin/pulp complex formation between Ag-BG/ECM treated group and control group. The Ag-BG/ECM treated group showed stronger DSP positive staining (**A**) and higher amounts of dentin formed (**C**) compared to the control group (**B**, **D** and **G**) (* = statistical significant difference compared to the control) ($p < 0.05$). Nestin staining was found in dental pulp cells in proximity to dentin-like matrix (De) (**F**) and signal was stronger in odontoblast-like cells (od) (**F**, **G**) and localised within their cells processes (arrows) (**G**) (Bar = 200 μm in **A-D** and **F**, 20 μm in **G**).

principal outcome of this work. The odontoblast-like cells lining the regenerated dentin-like tissue show also positive immunoreactivity for nestin (Fig. 7F-G) confirming their odontoblast-like phenotype.

Discussion

There is increasing interest in designing innovative treatment options to treat dental pulp infections with minimal removal of tooth structure and replacement with natural live tissue (Murdoch-Kinch and McLean, 2003). Moreover, it has been reported that the fabrication of innovative bioactive/biomimetic ion-releasing restorative materials, capable of re-mineralising the mineral-depleted dental tissues, has become one of the main targets in dental biomaterials research (Sauro *et al*., 2013). Thus, approaches using scaffolds and/or cells have been evaluated mainly for their regenerative potential in healing dental defective tissues (Ringe *et al*., 2002).

Enhanced and/or guided dentin tissue regeneration is an essential part needed for success of dental reparative

procedures. Obtaining sound reparative dentin will certainly maximise the outcome of dental procedures. The success of dentin/pulp regeneration for root canal treatments depends on the development of suitable scaffolding materials as carriers for DPSCs. Towards this long term goal, our study describes the fabrication and characterisation of a novel bioactive and injectable material Ag-BG/ECM with strong anti-bacterial properties and enhanced cell differentiation, mineralisation potential and dentin formation. Furthermore, this study shows that the integration of an Ag-doped silicate BG into the natural ECM hydrogel significantly enhanced DPSCs proliferation and differentiation. This approach is novel and promising, as it combines the synergetic properties of each component, leading to better biological, antibacterial and mineralisation activities of the composite Ag-BG/ECM scaffold than the individual components.

An important characteristic of the silicate bioactive glasses is their ionic degradation by products, which are soluble and have been observed to stimulate mineralised tissue formation through the activation of the expression of several genes associated with mineralised matrix deposition (Xynos *et al.,* 2000) Consequently, if the ion release process of the BG could be controlled, then this could be critical to ensure desirable biological responses. The Ag-BG presents controlled ion release profile. The ion dissolution was constantly observed from Ag-BG for over 4 weeks, and no apparent burst release was observed on the first (Chatzistavrou *et al.*, 2014).

DPSCs are odontogenic progenitor cells and have clonogenic abilities, rapid proliferation rates and multiple differentiation potentials (odontogenic, osteogenic and adipogenic), providing a suitable cell source for tissue regeneration (Gronthos *et al*., 2000). When DPSCs are used for pulp/dentin regeneration, their proliferation and odontogenic differentiation should be enhanced. An appropriate microenvironment that can facilitate cell-material interactions could be crucial for the achievement of these goals. Our data showed that when Ag-BG is incorporated into the ECM hydrogel, DPSCs had higher proliferation and lower apoptosis percentage compared to ECM alone and control, indicating that the ions released from the Ag-BG influence cellular proliferation and apoptosis. The released ions – such as calcium, phosphate and silicon – enhance differentiation and mineralisation of the cells through ion-mediated reactions, as also demonstrated in several other types of ion-eluting bioactive glass materials (Sachlos *et al*., 2003; Marelli *et al*., 2010). This action may also be important in triggering the odontogenic function of DPSCs. Indeed, we found that the expression levels of marker genes for odontogenic differentiation were all significantly higher in the Ag-BG/ECM than the control. This is not the case when ECM alone is compared to the control.

In particular, the odontoblastic activity of the DPSCs was evaluated by measuring their ALP activity, Alizarin red stain level and mRNA level of cell differentiation markers. ALP has been used to evaluate osteo/odontoblastic activity in various engineered biomaterials, as it is considered an important phenotype of the bone and dentin forming cells (Hong *et al*., 2009; Nam *et al.*, 2011). The influences of Ag-BG/ECM and ECM alone on the odontogenesis of DPSCs were examined by measuring the mRNA expression levels of ALP, RUNX2, DMP-1 and DSPP, which were chosen as markers for the odontogenic phenotype. The latter two genes are specific markers in odontogenesis as DMP-1 is accepted as key marker for odontoblasts in newly formed dentin (Narayanan *et al*., 2001) and DSPP is a well-known specific marker expressed by odontoblastic cells, while higher expression observed as dentin mineralisation progresses (Hanks *et al.*, 1998). RT-PCR data confirmed that DPSCs treated with Ag-BG/ECM had up-regulated mRNA levels for all markers used when compared to the other groups. These data show that the biomimetic Ag-BG/ECM provides better chemical composition and microenvironment that significantly enhances proliferation, differentiation and biomineralisation of DPSCs.

Ag-BG is a silicate-based glass with Ca/P ratio close to 4, capable of inducing apatite formation when immersed in simulated body fluid (SBF) due to the dissolution and subsequent protonation of the Si-O groups to form silanols as seats for heterogeneous nucleation and crystallisation (Kim *et al*., 2010). Measurements of the ion release in suspension demonstrated that Ag-BG releases ions into its environment (Chatzistavrou *et al*., 2014). Ag-BG can therefore be considered as an efficient calcium and silicon source for remineralisation processes. Based on our previous results (Chatzistavrou *et al*., 2012), which showed that Ag-BG enhances the development of hydroxyapatite phase, we also postulate that the new material Ag-BG/ECM will also induce apatite formation and consequently could develop strong bonds with the surrounding dentin. However, more research is necessary for the evaluation of this potential property combined with the evaluation of the mechanical properties of the developed bond.

Among other anti-bacterial agents, different forms of silver (Ag) have been previously incorporated into dental materials (Yoshida and Atsuta, 1999; Cheng *et al*., 2012; Zhang *et al.*, 2012). Although these strategies have shown various degrees of success in inhibiting bacterial growth, the resulting modified materials lack bioactive properties that can induce remineralisation of the mineral-depleted dental tissue, repair and regeneration. In our study, Ag ions were incorporated into a silicate bioactive glass and this was combined into ECM hydrogel, obtaining an injectable material that enhances endogenous cells response and provides bacterial protection. Indeed, our Ag-BG/ECM material shows complete bactericidal effects against *S. mutans* and *L. casei* growth. The antibacterial action of the Ag-BG/ECM could be only attributed to the released Ag ions from the Ag-BG and not to changes at the pH value. Our previous work (Chatzistavrou *et al*., 2014) presents in detail the ion release profile of this material and the calculated concentration of the released silver was higher than the minimum required for expressing antibacterial activity (0.1 ppm) and well below the cytotoxic levels for human cells (1.6 ppm) (Saravanapavan *et al*., 2004). Similarly, the pH value remains at neutral level (~ 7.5) during a soaking time period more than a month. We are not expecting different properties in case of Ag-BG/ECM as ECM hydrogel is also neutralised before the incorporation of Ag-BG. Our ongoing studies aim to further characterise

the anti-bacterial effects of Ag-BG/ECM using different biofilm assays.

There is extensive use of natural extracellular matrix scaffold (ECM) for regeneration of defected organs (Badylak, 2002; 2007). ECM scaffolds are being used extensively for generation of damaged tissues but also for whole organ regeneration (Badylak, 2004). However, it is still unclear if regeneration of defective tooth parts can be performed on a large scale (Mitsiadis and Papagerakis, 2011). In the short term, it may be more feasible to design and fabricate regenerative materials that enhance topically the regenerative potential of the defective dental tissues (Chatzistavrou *et al.*, 2012). Towards this goal, we have also tested the regenerative properties of ECM hydrogel. Our data show that ECM induces cell proliferation and cell differentiation. We also show that Ag-BG integrated into ECM scaffolds result in strong bacterial growth inhibition. Of interest, the combined Ag-BG/ECM shows also enhanced cell proliferation and differentiation properties in addition to the improved anti-bacterial action.

Previous experiments have shown that transplantation of DPSCs, mixed with hydroxyapatite/tricalcium phosphate, formed an ectopic pulp-dentin-like tissue complex in nude mice (Gronthos *et al.*, 2000). Our *in vivo* results show enhanced dentin and dental mineralising cells formation for those DPSCs treated with Ag-BG/ECM compared to the control group. These dental mineralising cells facing the newly formed matrix have the distinct phenotypic characteristic of odontoblasts expressing nestin and DSP. Therefore, we postulate that the released ions from the Ag-BG, combined with the released biomolecules of the natural ECM hydrogel and its inherent biological properties, may promote dentin regeneration. These data provide more evidence for the ability of this new material to promote odontogenic differentiation *in vivo*.

We therefore, postulate that Ag-BG/ECM may be used in the future for dental pulp treatment approaches, providing optimised dental pulp regeneration under bacteria-free conditions. The *in vivo* results indicate potential clinical applications of Ag-BG/ECM, as a dental material with unique properties of healing and regenerating dental tissues, while promoting bacteria-free conditions. Ag-BG can find applications in the restoration of dental lesions with direct or indirect pulp injury. This could significantly increase the lifetime of the restoration, leading to a breakthrough novel approach with superior outcome compared to the traditional currently used dental approaches. Even though, our studies highlight that Ag-BG/ECM demonstrates enhanced anti-bacterial and regenerative properties, while lacking any detectable toxicity, additional studies are necessary to examine in more detail the advantages of Ag-BG/ECM *in vivo*.

Conclusion

This study reports the fabrication and evaluation of novel bioactive and antibacterial material (Ag-BG/ECM) able to inhibit *S. mutans* and *L. casei* growth and to induce dental pulp cells proliferation and differentiation. The *in vivo* results support the potential use of Ag-BG/ECM in dental clinical applications with an emphasis on pulp treatment, as an injectable material for the restoration of lesions involving pulp injury. We believe that this initial study contributes to the field of regenerative dentistry, promoting minimally invasive approaches and provides foundation for future studies on Ag-BG/ECM material applications with clinical relevance.

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Disclosure of potential conflict of interest

The authors indicate no potential conflicts of interest.

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Editor's Note:

All questions/comments by the reviewers were answered by text changes. There is hence no Discussion with Reviewers section.