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Growth Factor Liberation and DPSC Response Following Dentine Conditioning

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Abstract

Liberation of the sequestrated bioactive molecules from dentine by the action of applied dental materials has been proposed as an important mechanism in inducing a dentinogenic response in teeth with viable pulps. Although adhesive restorations and dentine-bonding procedures are routinely practiced, clinical protocols to improve pulp protection and dentine regeneration are not currently driven by biological knowledge. This study investigated the effect of dentine (powder and slice) conditioning by etchants/conditioners relevant to adhesive restorative systems on growth factor solubilization and odontoblast-like cell differentiation of human dental pulp progenitor cells (DPSCs). The agents included ethylenediaminetetraacetic acid (EDTA; 10%, pH 7.2), phosphoric acid (37%, pH <1), citric acid (10%, pH 1.5), and polyacrylic acid (25%, pH 3.9). Growth factors were detected in dentine matrix extracts drawn by EDTA, phosphoric acid, and citric acid from powdered dentine. The dentine matrix extracts were shown to be bioactive, capable of stimulating odontogenic/osteogenic differentiation as observed by gene expression and phenotypic changes in DPSCs cultured in monolayer on plastic. Polyacrylic acid failed to solubilize proteins from powdered dentine and was therefore considered ineffective in triggering a growth factor–mediated response in cells. The study went on to investigate the effect of conditioning dentine slices on growth factor liberation and DPSC behavior. Conditioning by EDTA, phosphoric acid, and citric acid exposed growth factors on dentine and triggered an upregulation in genes associated with mineralized differentiation, osteopontin, and alkaline phosphatase in DPSCs cultured on dentine. The cells demonstrated odontoblast-like appearances with elongated bodies and long extracellular processes extending on dentine surface. However, phosphoric acid–treated dentine appeared strikingly less populated with cells, suggesting a detrimental impact on cell attachment and growth when conditioning by this agent. These findings take crucial steps in informing clinical practice on dentine-conditioning protocols as far as treatment of operatively exposed dentine in teeth with vital pulps is concerned.

Keywords: cell differentiation, dentinogenesis, esthetic dentistry, gene-expression, stem cell(s), pulp biology

Introduction

Dentine matrix is a reservoir of bioactive molecules that are sequestrated during dentinogenesis. The releasable growth factors from dentine provide a possible source of cell signaling molecules for initiating repair in trauma/injury situations. The process may involve stimulation of the secretory activity of preexisting odontoblasts or recruitment, proliferation, and subsequent differentiation of dental pulp progenitor cells (DPSCs) to "osteodentine"-forming cells (Smith and Sloan 2007).

Among the growth factors present in dentine extracellular matrix, transforming growth factor β1 (TGF-β1), bone morphogenetic proteins (BMPs), and vascular endothelial growth factor (VEGF) are believed to play crucial roles in wound healing and repair (Zhang et al. 2011; Pakyari et al. 2013; Alaee et al. 2014). Clinically applied materials such as calcium hydroxide, or $Ca(OH)_2$, mineral trioxide aggregate, as well as ethylenediaminetetraacetic acid (EDTA) and acidic etching agents have the ability to release growth factors from dentine matrix (Graham et al. 2006; Tomson et al. 2007; Ferracane et al. 2013; Galler et al. 2015) and may facilitate a dentinogenic response. Evidence shows that dentine conditioning by EDTA affects the fate of dental progenitor cells, promoting cell survival, attachment, growth, and differentiation (Galler et al. 2011; Trevino et al. 2011; Pang et al. 2014).

Relevant to adhesive restorative treatment, acidic agents are shown to expose and release bioactive molecules from mineralized tissues (Smith et al. 2011; Galler et al. 2015). However, the kinetics of growth factor exposure and release and the impact of dentine conditioning by various clinically applied agents on DPSC behavior have not been previously investigated. The aim of this study was to investigate the effect of phosphoric, citric, and polyacrylic acids, as compared with EDTA, on solubilization of growth factors from pulverized dentine and the impact of dentine surface conditioning on DPSC behavior. We hypothesize

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that there are no differences in the effects of conditioning agents used with regard to the assessed parameters.

Materials and Methods

Human caries–free third molars were collected from the oral surgery clinics of the School of Dentistry, Cardiff University, with ethical approval (07/WSE04/84, Wales REC1) and patients' consent.

Extraction of Dentine Matrix Components by Conditioning Agents

Dentine powder was prepared as described in Appendix Figure 1. Equal amounts of dentine powder (4 g) were extracted with one of the following: 10% EDTA (conditioner for smear layer removal; positive control), 37% phosphoric acid (etchant used with composite restorations), 10% citric acid, or 25% polyacrylic acid (conditioner and constituent of glass ionomer restorations). Dentine matrix extract (DME) yield was calculated (w/w%) relative to dentine powder.

Protein Separation, Growth Factor Detection, and Quantification in DMEs

Protein concentrations in DME samples were determined with a Pierce BCA assay kit, and 30- and 150-µg DMEs (for silver staining and electroblotting, respectively) were loaded onto 4% to 15% mini-gels (Bio-RAD) for SDS-PAGE. A Silver Stain Kit (Pierce, Life Technologies) was used for protein detection. Western blot was performed with a semidry transfer cell (Bio-Rad), and immunodetection was achieved through primary antibodies, polyclonal rabbit anti-TGF-β1 and anti-VEGF (1:100) and goat anti-BMP2 (1:300), secondary HRP conjugated antibodies (1:3,000 to 1:10,000; Santa Cruz Biotechnology Inc.), and Hyperfilm ECL (GE Healthcare). Enzyme-linked immunosorbent assay (ELISA) kits were used for quantifying TGF-β1 (eBiosciences), BMP2, and VEGF in DMEs (Peprotech). Each extract was assayed in triplicate on 2 separate occasions. Mean values were analyzed through 1-way analysis of variance (ANOVA) with post hoc Tukey's honest significant difference (HSD) based on SPSS 20 (IBM Corp).

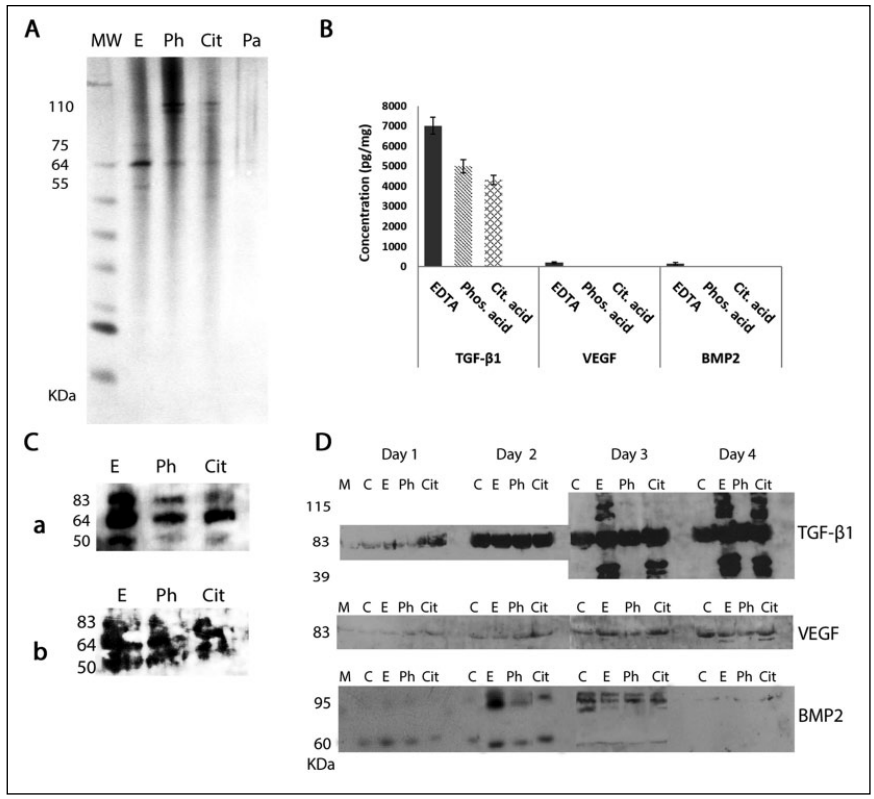

Figure 1. Protein separation, growth factor detection and quantification in dentine matrix extracts (DMEs), and expression of growth factors by DME-stimulated dental pulp progenitor cells (DPSCs). (**A**) Silver-stained gel illustrates the banding patterns representing proteins with different molecular weights in various samples: molecular weight marker (MW), DME from ethylenediaminetetraacetic acid (EDTA; E), phosphoric acid (Ph), and citric acid (Cit). No clear bands could be identified in the polyacrylic acid extracts (Pa). (**B**) Average concentrations of growth factors (pg/mg) in DMEs extracted by different agents as quantified by ELISA (n = 6; error bars represent standard error). (**C**) Western blots showing the presence of (a) TGF-β1 and (b) VEGF in DME extracted by EDTA, phosphoric acid, and citric acid. BMP2 could not be detected with this method. (**D**) Stimulation of DPSCs by DMEs appears to result in a sequential upregulation in growth factor expression in a 4-d period. Western blot showing growth factors in lyophilized conditioned media. BMP2 appeared to be expressed early on but is barely detectable by day 4. Stimulation by EDTA- and citric acid–extracted DMEs resulted in a more potent stimulatory response in DPSCs (as evidenced by remarkably higher expression of TGF-β1 and VEGF) compared with control (C) as well as cells exposed to phosphoric acid–extracted DMEs over the period of experiment. BMP2, bone morphogenetic protein 2; M, lyophilized media with no cells; TGF-β1, transforming growth factor β1; VEGF, vascular endothelial growth factor.

Influence of DMEs on DPSCs

A DPSC population (characterized by Lee et al. 2015) between passages 13 and 14 was used to investigate the impact on cell behavior for up to 21 d of stimulation with DMEs. Cells were seeded in 6-well plates at 2,400 cells/cm^2 and cultured in α-MEM, 10% heat-deactivated fetal bovine serum (FBS), 100 U/mL of penicillin G sodium, 0.1 µg/mL of streptomycin sulphate, and 0.25 µg/mL of amphotericin (all Gibco), and 100µM L-ascorbate 2-phosphate (Sigma-Aldrich) at 37 °C with 5% CO_2. After 24 h, cells were serum starved for 24 h, followed by addition of 3.5% FBS and 10 µg/mL of DMEs to media (optimal concentration determined by Lee et al. 2015). For negative controls, no DME or denatured EDTA-extracted DMEs (as in

Lee et al. 2015) were added, and mineral nodule formation in cultures was assessed.

Alizarin red (Sigma-Aldrich; 2% w/v, pH 4.2) and oil red O (Sigma-Aldrich; 0.23% w/v) staining identified mineral deposition and adipogenic differentiation, respectively, under a light microscope. Immunohistochemistry identified matrix proteins, collagen type 1, and DSP on days 2 and 14 post-DME stimulation. Cultures were fixed in 4% paraformaldehyde and exposed to 1:50 polyclonal primary antibodies, followed by FITC goat anti-rabbit (1:250) secondary antibodies (all Santa Cruz). Growth factor expression by cells following stimulation by DMEs was assessed through Western blot.

Treatment of Dentine Surfaces by Conditioning Solutions and Growth Factor Quantification

Dentine slices were immersed in the conditioning solutions for 5 or 10 min as detailed in Appendix Figure 1. Phosphate-buffered saline and 0.02M calcium hydroxide served as negative and positive controls, respectively. Solutions were removed and immediately frozen at −20 ºC for subsequent quantification of growth factors by ELISA (as described above). Dentine slices were washed thoroughly in distilled water and air-dried prior to immunogold localization. For scanning electron microscope (SEM) investigation of cell morphology and RNA extractions, dentine slices of approximately 0.8 to 1 cm in diameter were conditioned for 5 min (details in Appendix Fig. 1) prior to DPSC seeding. Experiments were repeated on one further occasion.

Immunogold Localization of Growth Factors on Conditioned Dentine Slice

Conditioned dentine slices were incubated with polyclonal rabbit anti-TGF-β1, mouse anti-BMP2, or VEGF (1:100; Santa Cruz) primary antibodies and gold-labeled secondary antibodies (1:100, 30 nm; BBI Diagnostics), followed by silver-enhancing agent (BBI Diagnostics) prior to carbon coating and visualization under SEM. Gold-labeled particles were counted for 5 random fields of 20 μm^2 per slice on 3 dentine slices. Mean values were analyzed with 1-way ANOVA with post hoc Tukey HSD.

Influence of Conditioned Dentine Slices on Behavior of DPSCs

DPSCs were cultured on conditioned dentine slices (4.3×10^4 cells/cm^2) and cultured in media containing 7% FBS as described above. Morphology of cells was examined by SEM after 1 and 8 d. Slices were washed with phosphate-buffered saline and fixed in 2% paraformaldehyde and 2.2% glutaraldehyde in 0.1M cacodylate buffer, then rinsed in cacodylate buffer, dehydrated in graded ethanol, and air-dried prior to carbon coating and SEM analysis.

Gene Expression Analysis

Changes in gene expression with DME stimulation in cells cultured on plastic or conditioned dentine surfaces were investigated. Total RNA was extracted with the Qiagen RNeasy Mini Kit (Qiagen Ltd). cDNA was synthesized with 1 μL of Moloney murine virus reverse transcriptase, 0.625 μL of RNasin, and 1.25 μL of dNTPs (all Promega) at 37 ºC for 1 h. Reverse transcription polymerase chain reaction (RT-PCR) was performed to identify the expression of stem cell, proliferation, and differentiation marker genes via forward and reverse primers (Table) and GoTaq DNA polymerase over 40 cycles. Beta-actin served as the housekeeping gene. RT-PCR products were separated on 2% agarose gels containing ethidium bromide and visualized under ultraviolet light. Quantitative polymerase chain reaction (qPCR) based on SYBR Green Jumpstart Taq quantified gene expression in DPSCs following DME stimulation (*CD105*, proliferating cell nuclear antigen [*PCNA*], *RUNX2*, alkaline phosphatase [*ALP*], and osteopontin [*OPN*]) and dentine slice conditioning (*CD105, ALP*, and *OPN*; primers listed in Table). Each cDNA sample was analyzed in triplicate by qPCR on triplicate samples for each condition. Based on the comparative cycle threshold method (ΔΔCt), mean values were calculated as percentage expression relative to *GAPDH* (housekeeping gene) and analyzed through 1-way ANOVA and post hoc Tukey HSD.

Results

Characterization of DMEs

DME yields were 1.4%, 3.3%, and 0.8% relative to dentine powder for EDTA, phosphoric acid, and citric acids, respectively. Differences were observed in the protein profiles of the variously extracted DMEs. For polyacrylic acid, the weight of lyophilized extracts was more than the initial dentine powder; no band formation was identified in the gel with silver staining. Western blots identified TGF-β1 and VEGF in EDTA, phosphoric acid, and citric acid DMEs. BMP2 could not be detected through this method. ELISA identified the highest concentrations of TGF-β1 in EDTA DMEs. BMP2 and VEGF were detected in EDTA DMEs (Fig. 1A, B).

Influence of DME Stimulation on DPSCs

Calcium-containing nodules were apparent in DME-stimulated DPSC cultures but not in control on day 8 (Appendix Fig. 2). Mineralization became widespread in all DME-stimulated groups by day 21; controls showed faint and sparse mineral deposition (Fig. 2A). On day 21, random lipid-containing cells were detected in control, but there was no obvious evidence of adipogenesis in DME-stimulated groups (Appendix Fig. 2). Collagen 1A1 and DSP were detected by immunocytochemistry in all DME-stimulated cultures on day 2 (Appendix Fig. 2) with levels increasing on day 14 and demonstrating more widespread immunofluorescence as compared with control (Fig. 2B, C).

Table. Human Primer Sequences and Annealing Temperatures for RT-PCR and qPCR.

Gene Marker	Product Size, bp	Annealing Temperature, °C	Sequence, 5′ 3′
		RT-PCR	
ALP	613	68	TGTGCCTGGACGGACCCTCGCCAGTGCT CCAGCGTCCTTGGCCCAGCGCAGGATGG
β-actin	480	62	AGGGCAGTGATCTCCTTCTGCATCCT CCACACTGTGCCCATCTACGAGGGGT
CD105	483	55	GAAGGGCTGCGTGGCTCAGG CCTTCCAAGTGGCAGCCCCG
CD73	351	65	GTCGCGAACTTGCGCCTGGCCGCCAAG TGCAGCGGCTGGCGTTGACGCACTTGC
CD90	425	55	ATGAACCTGGCCATCAGCATC CACGAGGTGTTCTGAGCCAGC
Collagen 1A1	401	68	AGCAGGAGGCACGCGGAGTGTGAGGCCA TCGGTGGGTGACTCTGAGCCGTCGGGGC
PCNA	571	53	GGCGTGAACCTCACCAGTAT CTTCATCCTCGATCTTGGGA
PPARG2	349	60	GCCATCAGGTTTGGGCGGATGCCACAG CCTGCACAGCCTCCACGGAGCGAAACT
		qPCR	
ALP	137	55	GGACCATTCCCACGTCTTCAC CCTTGTAGCCAGGCCCATTG
CD105	186	55	AACAGTCCATTGTGACCTTC TTTTGCTTGGATGCCTGG
GAPDH	253	55	GGTCGGAGTCAACGGATT ATCGCCCCACTTGATTTTG
OPN	430	55	ATCACCTGTGCCATACCA CATCTTCATCATCCATATCATCCA
PCNA	184	55	CTCTTCAACGGTGACACTC TGGCATCTTAGAAGCAGTTC
RUNX2	105	62	CGCCTCAGAACCCACGGCCC ACGGCGGGGAAGACTGTGCC

bp, base pairs; qPCR, quantitative polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

RT-PCR revealed *CD105, CD90, CD73, PCNA*, and *collagen 1* gene expression between days 1 and 21. *ALP* expression appeared on day 14 in DME-stimulated groups. Adipogenic marker *PPARγ2* gene expression was detected in control but not in DME-stimulated groups (Fig. 2D).

The qPCR revealed lower expression of *CD105* in DME-stimulated groups compared with control ($P < 0.05$, day 21). *PCNA* was upregulated between days 3 and 21 in control ($P < 0.05$, compared with test groups on day 21). *RUNX2* was expressed in all groups ($P > 0.05$, between groups). *OPN* was significantly upregulated with DME stimulation as compared with control ($P < 0.01$, day 21). *ALP* was upregulated with DME stimulation ($P < 0.05$, for groups stimulated with EDTA and phosphoric acid DMEs, day 21; Fig. 2E).

Growth Factor Exposure and Release from Conditioned Dentine Slices

Immunogold localization revealed that all the conditioning agents exposed TGF-β1, BMP2, and VEGF on dentine surfaces as represented by the gold-labeled particles (Fig. 3A). The mean value of particle counts indicated a drop in surface levels of TGF-β1 between 5 and 10 min with $Ca(OH)_2$ conditioning. Conditioning with EDTA, phosphoric acid, and citric acid resulted in a slight increase in TGF-β1 exposure over time. BMP2 and VEGF exposure increased slightly between 5 and 10 min with $Ca(OH)_2$ conditioning, while a decrease was observed with EDTA, phosphoric acid, and citric acid.

ELISA detected the release of TGF-β1 into solution only with EDTA conditioning ($P < 0.01$). Citric acid appeared to be the most effective agent for BMP2 and VEGF release after 5 min of dentine conditioning ($P < 0.01$; Fig. 3B, C).

Gene Expression and Morphology of DPSCs Cultured on Conditioned Dentine

Q-PCR data revealed a downregulation in *CD105* between days 2 and 10 in all groups, while *ALP* and *OPN* were upregulated between days 5 and 14. On day 14, DPSCs cultured on EDTA- and citric acid–treated dentine showed the highest level of *ALP* expression ($P < 0.01$ and $P < 0.05$, respectively). *OPN* expression was significantly higher in cells cultured on $Ca(OH)_2$-conditioned dentine on day 5 and on EDTA-conditioned dentine on day 14 (Fig. 4A).

SEM on day 2 revealed an abundance of cells on conditioned dentine slices. Overlapping cell bodies prevented a reliable quantification. The cell coverage was more obvious against the open dentinal tubules in the background with EDTA- and acid-conditioned surfaces as compared with $Ca(OH)_2$. On day 8, scarcity of cells on phosphoric acid–treated dentine was

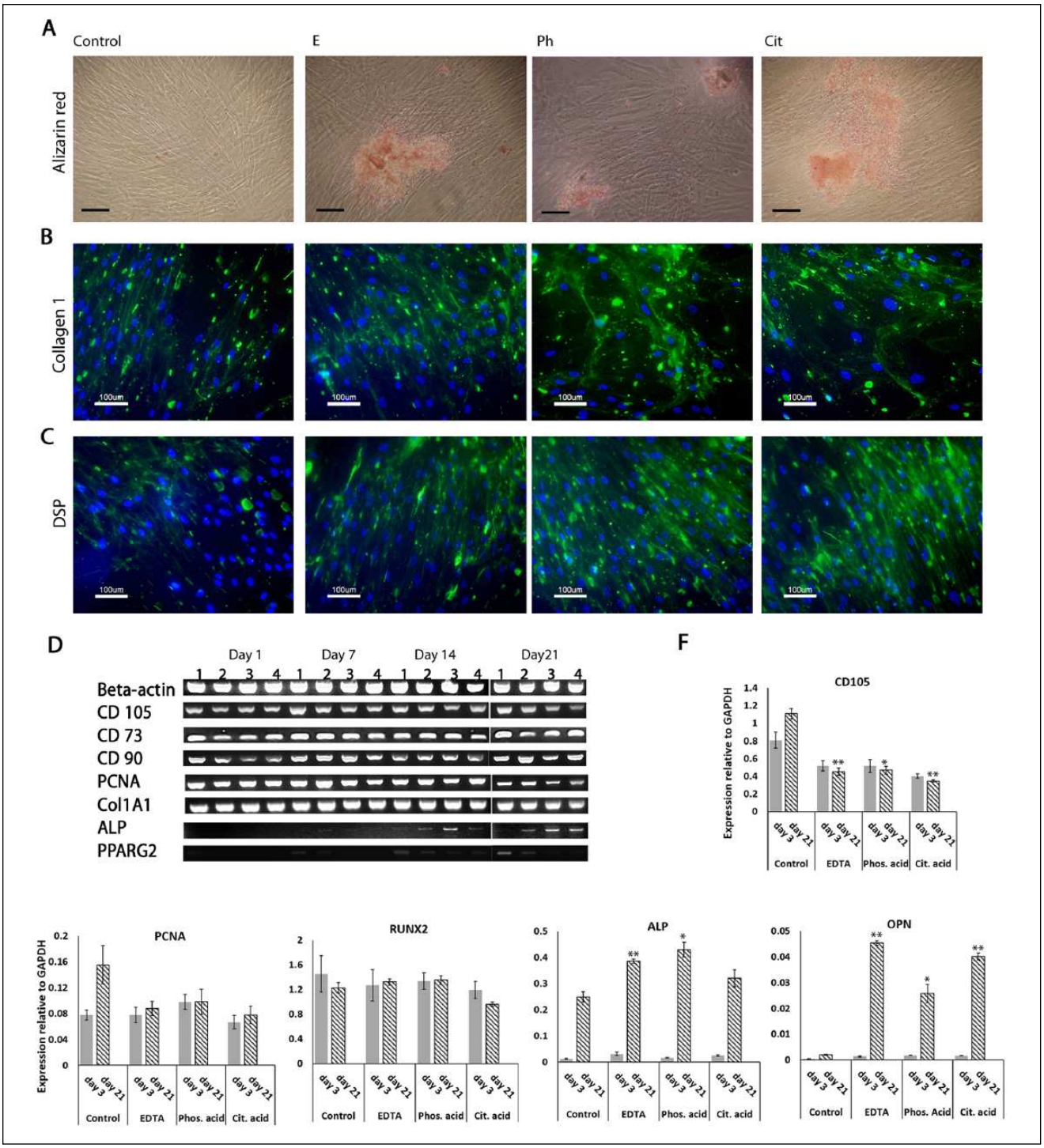

Figure 2. Mineralized nodule formation, matrix protein production, and dental pulp progenitor cell gene expression following stimulation by dentine matrix extracts (DMEs). (**A**) The effect of DME in inducing mineralized phenotype differentiation in dental pulp progenitor cells. Alizarin red staining reveals negligible mineralization in control as compared with test groups stimulated with ethylenediaminetetraacetic acid (EDTA; E), phosphoric acid (Ph), and citric acid (Cit) extracted DMEs (day 21; scale bar, 100 µm). Immunohistochemistry demonstrates expression of extracellular matrix proteins in dental pulp progenitor cell culture: (**B**) collagen type 1 and (**C**) DSP in control and DME-stimulated groups after 14 d in culture. Visual inspection of cultures under fluorescent microscope suggests more widespread fluorescence in DME-stimulated groups. Mounting medium containing DAPI (Vector Laboratories Inc.) identifies nuclei (scale bar, 100 µm). (**D**) Reverse transcription polymerase chain reaction analysis demonstrates the expression of mesenchymal, developmental, proliferation, and differentiation markers. With DME stimulation, the expression of mineralization marker alkaline phosphatase (*ALP*) is clearly detected from day 14. In the absence of DME stimulation in the control group, the expression of *PPARγ2*, however faint, can be detected on day 14. This is consistent with phenotypic changes observed (Appendix Fig. 2). (**F**) Quantitative polymerase chain reaction reveals a significant upregulation in differentiation marker genes *ALP* and osteopontin (*OPN*) with DME stimulation as compared with control, while the opposite was true for the stem cell marker *CD105* (the *y*-axis represents percentage expression relative to *GAPDH*). Comparative expression levels are also shown for proliferation marker *PCNA* (proliferating cell nuclear antigen) and transcription factor *RUNX2*. $^{*}P < 0.05$ and $^{**}P < 0.01$ ($n = 3$, error bars represent standard error).

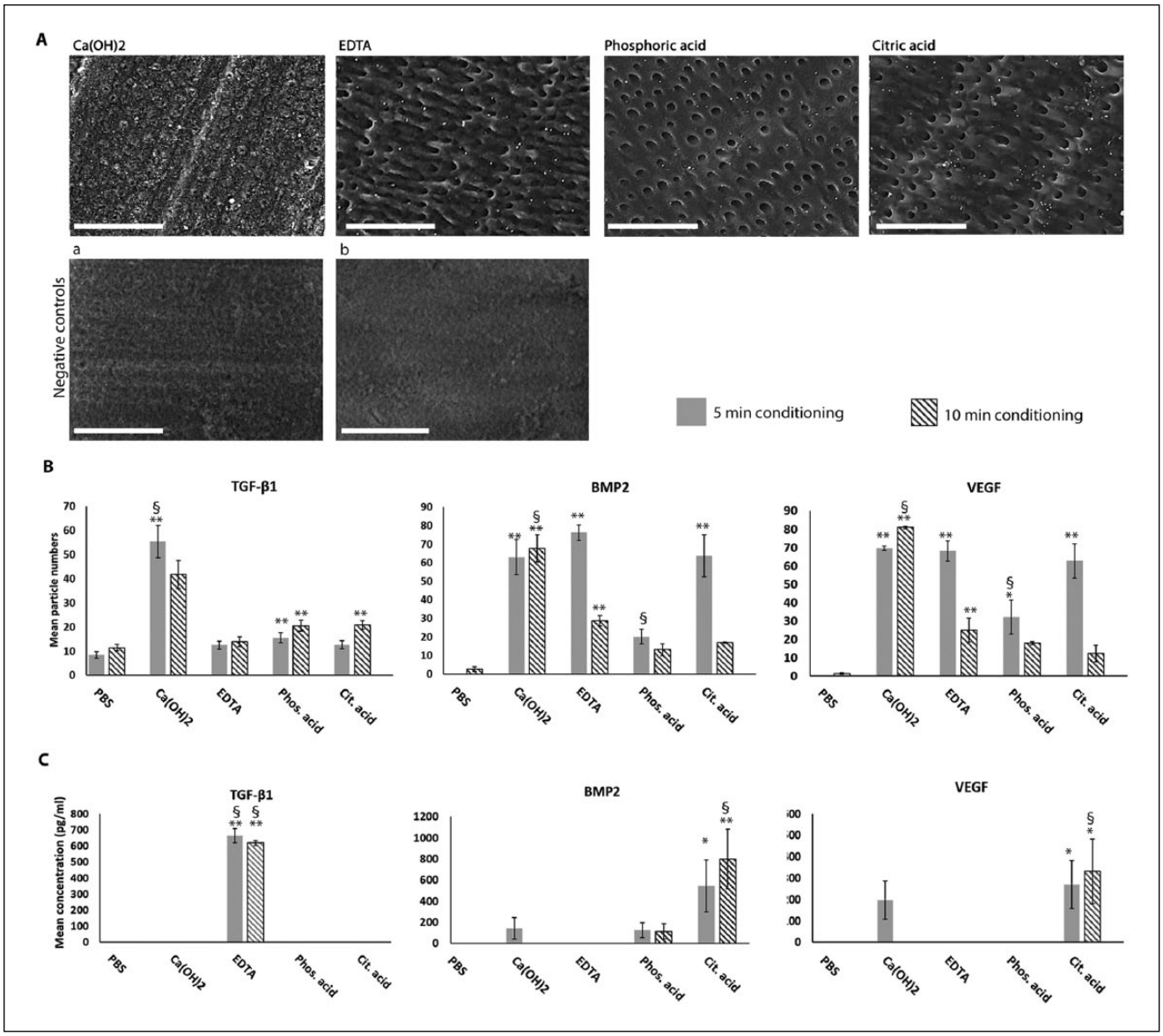

Figure 3. Growth factor exposure and release following dentine slice conditioning. (A) Representative scanning electron microscope images of immunogold-labeled TGF-β1 on dentine surfaces conditioned for 5 minutes with $Ca(OH)_2$ (calcium hydroxide), ethylenediaminetetraacetic acid (EDTA), phosphoric acid, and citric acid (magnification ×750; scale bar, 20 μm). For negative controls, primary antibodies were withdrawn or blocked with 10× excess of the blocking peptide (Santa Cruz) applied to slices treated with $Ca(OH)_2$ (a, b). (**B**) Graphs represent average counts of immunogold-labeled particles per 20 μm^2 of treated dentine surface (n = 15, error bars represent standard error). (**C**) Graphs represent average concentrations of growth factor in the supernatants of conditioned dentine as quantified by ELISA (n = 6, error bars represent standard error). *P < 0.05 and **P < 0.01, significant difference with PBS-treated dentine. §Significant difference with the remaining groups at P < 0.05. BMP2, bone morphogenetic protein 2; PBS, phosphate-buffered saline; TGF-β1, transforming growth factor β1; VEGF, vascular endothelial growth factor.

striking. Comparatively, dentine surfaces conditioned with $Ca(OH)_2$, EDTA, and citric acid showed a uniform and thick coverage by cells and extracellular substance (Fig. 4B–E).

Discussion

This in vitro study investigated the biological impact of dentine conditioning by agents relevant to adhesive restorative systems, focusing on a growth factor–mediated response from human DPSCs. Our results demonstrated that a presumed liberation of growth factors from dentine appears to correlate with deposition of a mineralized matrix by DPSCs.

Different conditioning agents extracted a different amount and profile of protein from dentine powder. The differences observed are significant, as dentine matrix components are known to induce tertiary dentine formation below injury sites, protecting the pulp from further insult (Smith et al. 1995; Smith et al. 2001). TGF-β1 and VEGF were detected in DMEs extracted from dentine powder by EDTA, phosphoric acid, and citric acid. BMP2 was detected in DMEs of EDTA but not

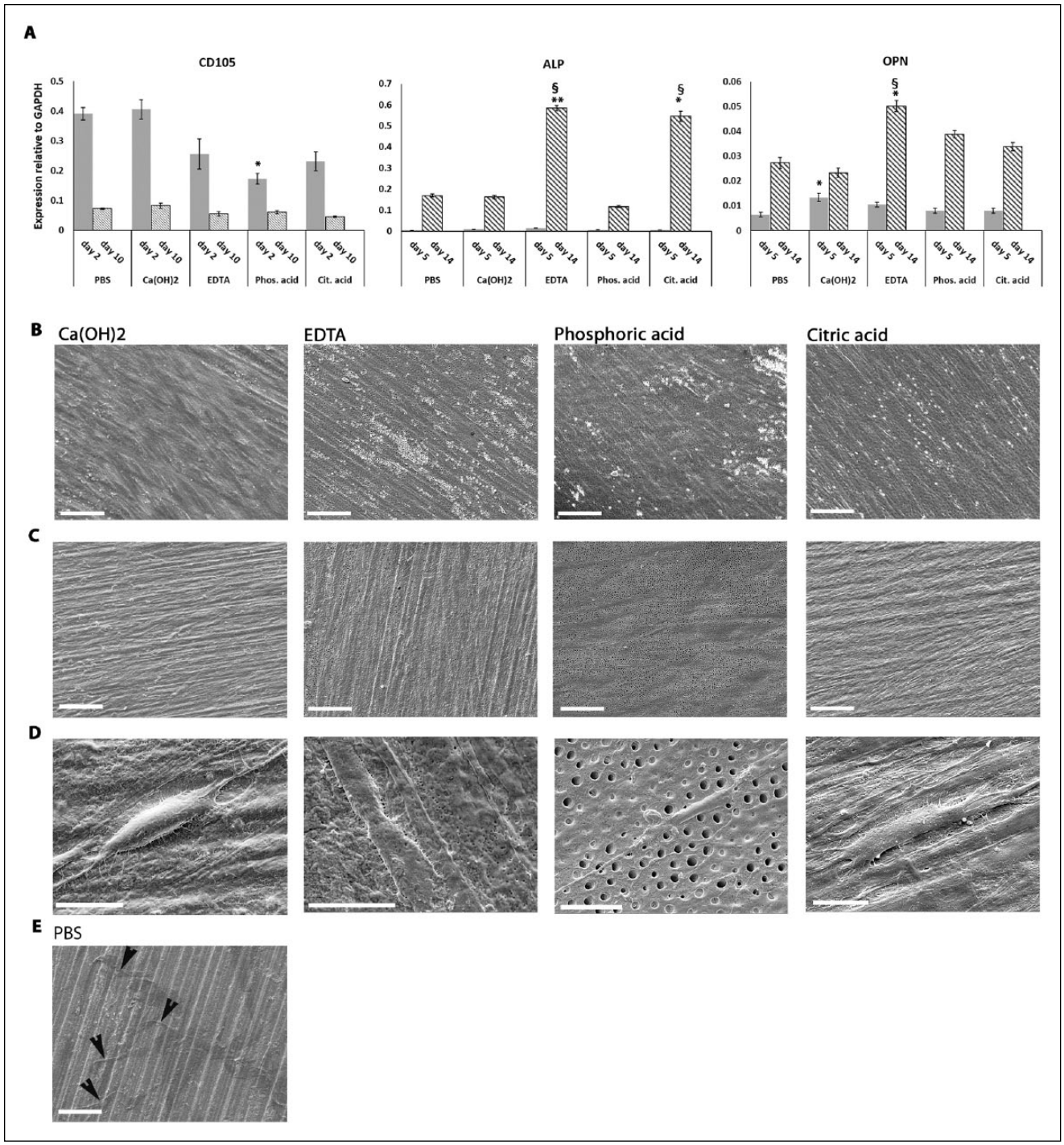

Figure 4. Dental pulp progenitor cell (DPSC) morphology and gene expression when cultured on conditioned dentine slices. (**A**) Gene expression of DPSCs cultured on conditioned dentine surfaces (the *y*-axis represents percentage expression relative to *GAPDH*). Between days 2 and 10, a downregulation in the expression of stem cell marker *CD105* is evident in all groups. An opposite trend in the expression of differentiation marker genes alkaline phosphatase (*ALP*) and osteopontin (*OPN*) is observed between day 5 and 14. **P* < 0.05 and ***P* < 0.01, significant difference with cells cultured on PBS-treated dentine. §Significant difference with the remaining groups at *P* < 0.05 (*n* = 3, error bars represent standard error). Representative scanning electron microscope images of DPSCs on dentine surface treated with $Ca(OH)_2$ (calcium hydroxide), ethylenediaminetetraacetic acid (EDTA), phosphoric acid, and citric acid. A subjective comparison of cell density could be made in lower magnification images on (**B**) day 2 and (**C**) day 8 (all ×500; scale bar, 100 µm). Although the outline of individual cells is less obvious on dentine surfaces treated with $Ca(OH)_2$ against the smear layer–covered surface, denser cell coverage is apparent on $Ca(OH)_2$, EDTA, and citric acid as compared with phosphoric acid–treated dentine on day 8. (**D**) Larger magnification on day 8 confirms a denser coverage on surfaces treated with the above-mentioned agents when compared with phosphoric acid (×4K, 5K, 3.5K, and 3.3K magnification; scale bars, 20 µm). (**E**) Sparse cell coverage on phosphate-buffered saline (PBS)–treated dentine, with few cells visible (arrows) on the smear layer–covered dentine (day 8, ×500; scale bar, 100 µm).

phosphoric or citric acid, probably due to degradation by strong acids; EDTA (neutral pH) chelates calcium ions from dentine matrix with little known impact on proteins (Graham et al. 2006; Smith et al. 2011).

Polyacrylic acid failed to extract proteins from dentine powder, with our yield data suggesting formation of insoluble complexes due to acid-protein conjugation (Bhattacharjee et al. 2012). Within a clinical situation, this may result in immobilization of dentin matrix proteins at sites of glass ionomer application. With the apparent inability of polyacrylic acid to liberate growth factors from dentine, its use was abandoned in the subsequent experiments. However, the above observations of polyacrylic acid are relevant to the clinical dilemma of whether to directly "bond" a composite restoration in deep cavities or to "base" it with glass ionomer cement. Evidence is currently inconclusive on the comparative superiority of the sandwich technique versus direct composite resin restorations for microleakage control (Kasraei et al. 2011; Gungor et al. 2014; Moazzami et al. 2014). Our findings highlight an important aspect where potential differences exist between the 2 techniques, shifting the balance against the apparently inert glass ionomer system in situations where inducing a reparative response is crucial for the treatment success.

With regard to the impact of DMEs on cell behavior, this study found EDTA, phosphoric acid, and citric acid DMEs to be potent stimulators of odontoblast/osteoblast-like differentiation. The sporadic mineralization and random adipogenic differentiation in control cultures on day 21 may be explained by increased cell numbers and cellular contacts as well as paracrine signaling (Tang et al. 2010; Zhurova et al. 2010). Several cell signaling molecules, including BMPs and insulin growth factor, have been proposed to display pro-osteogenic and pro-adipogenic effects (James 2013). TGF-β1, BMP2, and VEGF were detected in the conditioned media of DPSCs over a 4-d culture period. DME stimulation, in particular by EDTA and citric acid DMEs, resulted in upregulation of growth factor production, which may be important in inducing differentiation via downstream signal transduction.

The DPSC clone used here expressed characteristic mesenchymal stem cell markers. Opposing trends in the expression of *ALP* (mineralizing differentiation marker) and *PPARγ*$_2$ (adipogenic marker) in DME-stimulated groups versus control suggest that DMEs stimulated DPSCs toward mineralized differentiation and reduced adipogenic differentiation. Q-PCR revealed an upregulation in *PCNA* expression in control between days 3 and 21, suggesting ongoing proliferation while the DME-stimulated groups had moved on in their stage of development, as evidenced by a significant upregulation in differentiation markers *ALP* and *OPN*, mineral deposit formation, and earlier detection of DSP in DME-stimulated cultures.

We also investigated growth factor exposure and release following dentine slice conditioning. Preliminary data (not shown) revealed little growth factor exposure with 2 min of conditioning, and for a conclusive comparison between groups, treatment times were increased to 5 and 10 min. Dentine conditioning with citric acid for 5 min resulted in BMP2 and VEGF exposure in levels comparable with positive controls, $Ca(OH)_2$ and EDTA and significantly more than phosphoric acid. This is an interesting finding, bearing in mind that BMP2 and VEGF were not identified in citric acid–extracted DMEs. The extraction of DMEs over 2 wk with constant exposure to the acid may have resulted in denaturing of some proteins.

Citric acid was found significantly more effective than EDTA and phosphoric acid in releasing BMP2 and VEGF into the aqueous environment. Growth factor exposure and release are highly relevant to vital pulp treatments. In dentine cavities, dentinal tubules act as channels of communication between the cavity base where the conditioner is applied and the pulp tissue beneath. Growth factor exposure on dentine at the interface with pulp may affect the behavior of cells in contact with it (preexisting odontoblasts), while the released growth factors may diffuse into the pulp, promoting differentiation toward odontoblast-like cells (Tziafas et al. 2000; Smith et al. 2001). We acknowledge that the dentine-conditioning times here were much longer as compared with clinical protocols. The findings, however, highlight the comparative effect of various conditioning agents and provide a useful basis for future clinical studies and the optimizing of clinical protocols.

Analysis of gene expression and behavior of DPSCs cultured on conditioned dentine demonstrated, with culture time, downregulation of *CD105*, concurrent with upregulation in *ALP* and *OPN*, suggesting loss of stem cell characteristics and progression toward differentiation. The highest level of *OPN* expression on day 5 observed with $Ca(OH)_2$ conditioning may indicate a more advanced differentiation status as compared with other test groups. The highest level of *ALP* expression observed with cells cultured on EDTA- and citric acid–treated dentine may indicate advancing differentiation in these groups by day 14. The observation by SEM of a denser cell coverage—which appeared stretched and anchored on dentine surfaces by fibers extending onto an extracellular-like substance—reaffirmed a more favorable biological response with $Ca(OH)_2$, EDTA, and citric acid conditioning as compared with phosphoric acid treatment. The striking scarcity of cells on phosphoric acid–treated dentine suggests that it was not as supportive for cell attachment and growth. This may be detrimental in vital pulp treatments, as cell attachment to dentine precedes proliferation and differentiation to odontoblast-like cells and dentine regeneration and repair (Ring et al. 2008; Pang et al. 2014). The differences observed in cell behavior on dentine treated by different agents may be related to not only chemical but also physical properties of the surface, such as topography (Tejeda-Montes et al. 2014) and the diameter of dentinal tubules (Osorio et al. 2010). Substrate topography has been shown to influence stem cell behavior, such as adhesion, proliferation, and differentiation (Dalby et al. 2007; Kolind et al. 2014). Other properties (e.g., hardness of the thin residual dentine layer) may vary following demineralization, which can also influence cell behavior (Fu et al. 2010). Furthermore, strong acids can break down proteins (West et al. 2000) and damage the ultrastructure of macromolecules, such as collagen type 1 (Breschi et al. 2003), which may disrupt the adhesion motifs

for cell binding via integrin receptors (Heino and Kapyla 2009). In addition, the direct effect of conditioning agent on cells should not be overlooked. Even a 0.37% concentration of phosphoric acid can cause marked decrease in rat pulp cell viability (Nassar et al. 2013). Conversely, substances leached from a clinically applied $Ca(OH)_2$ cement were biocompatible for human pulp cells in vitro (Cavalcanti et al. 2005).

In summary, we observed that 10% EDTA and 10% citric acid appeared to be considerably better conditioners than 37% phosphoric acid for triggering a biological response in DPSCs. As for the implications for clinical practice, considerations should be given to replacing 37% phosphoric acid for dentine etching in adhesive restorative systems. This is particularly crucial when restoring deep cavities in vital teeth. Although concerns have been expressed in the past regarding the capacity of milder acids for etching enamel (Goracci et al. 2004; Brackett et al. 2006), in agreement with our view, EDTA has been proposed for etching dentine and enamel as a substitute for phosphoric acid in a recent study (Imbery et al. 2012). Such an approach may offer the added benefit of bond stability with deactivating MMPs and inhibiting the breakdown of the bond interface.

The findings of this study take crucial steps away from a merely mechanistic approach and toward a biological one in the management of dentine cavities. However, further research is required for optimizing dentine-conditioning protocols and developing strategies for promoting dentine regeneration in response to a placed restoration, to improve treatment predictability and longevity of teeth with compromised dental pulps.

Author Contributions

L. Sadaghiani, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H.B. Gleeson, S. Youde, contributed to data acquisition, critically revised the manuscript; R.J. Waddington, contributed to design, critically revised the manuscript; C.D. Lynch, contributed to conception, critically revised the manuscript; A.J. Sloan, contributed to conception and design, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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