

ORIGINAL ARTICLE

Growth factor release and dental pulp stem cell attachment following dentine conditioning: An *in vitro* study

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Abstract

Aim: The aim of the study was to investigate the effect of dentine conditioning agents on growth factor liberation and settlement of dental pulp progenitor cells (DPSCs) on dentine surfaces.

Methodology: The agents used 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). Human dentine slices were conditioned for exaggerated conditioning times of 5 and 10 min, so that the growth factor liberation reached quantifiable levels above the limit of detection of the laboratory methods employed. Transforming growth factor beta-1 (TGF- β 1) release and surface exposure were quantified by enzyme-linked immunosorbent assay (ELISA) and immunogold labelling. Scanning electron microscopy (SEM) was used to assess the morphology of cells and coverage by DPSCs cultured on dentine surfaces for 8 days.

Results: After 5-min conditioning of dentine slices, citric acid was the most effective agent for growth factor release into the aqueous environment as measured by ELISA (Mann–Whitney *U* with Bonferroni correction, $p < .01$ compared with phosphoric and polyacrylic acid). As well as this, dentine slices treated with phosphoric acid for the same period, displayed significantly less TGF- β 1 on the surface compared with the other agents used, as measured by immunogold labelling (MWU with Bonferroni correction, $p < .05$). After 8 days, widespread coverage by DPSCs on dentine surfaces conditioned with citric acid and EDTA were evident under SEM. On dentine surfaces conditioned with phosphoric and polyacrylic acid, respectively, less spread cells and inconsistent cell coverage were observed.

Conclusions: Based on the findings of this *in vitro* study, a desirable biological growth factor-mediated effect may be gained when conditioning dentine by milder acidic or chelating agents such as citric acid and EDTA. The results must be interpreted in the context that the potential of the applied materials inducing a desirable biological response in DPSCs is only one consideration amongst other important ones in a clinical setting. However, it is crucial to look beyond the mere physical effects of materials and move towards biologically based treatment approaches as far as the restorative management of teeth with viable dental pulps are concerned.

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KEYWORDS

acid etching, reparative response, TGF-beta 1, vital pulp

INTRODUCTION

The increased patient demand for more aesthetic restorations combined with concerns over the environmental impact of dental amalgam has resulted in adhesive restorations gaining popularity in recent decades. The conservative cavity preparation required makes adhesive restorations a popular choice for dental professionals as well, which has resulted in a rapid evolution in dental adhesive technology (Van Meerbeek et al., 2020). The current clinical protocols for dentine bonding, however, are not driven by biological understanding.

Dentine matrix contains a cocktail of growth factors with potent biological effects that have been secreted largely by odontoblasts during odontogenesis (Sloan et al., 2000; Smith, 2003). After secretion, the growth factors interact with other extracellular matrix components and thereby become embedded within dentine matrix, a process which is important for retaining their biological activity (Baker et al., 2009; Sloan et al., 2002; Smith et al., 1998).

Current evidence suggests that a range of materials relevant to the clinically applied agents, such as acidic and chelating dentine conditioners and etchants, as well as the alkaline $\text{Ca}(\text{OH})_2$ and MTA, can liberate the sequestered bioactive molecules from dentine matrix (Ferracane et al., 2013; Graham et al., 2006; Tomson et al., 2007). The acidic and chelating agents can cause localized demineralization, exposure of collagen fibrils and release of soluble bioactive molecules from dentine matrix (Ferracane et al., 2013; Pang et al., 2014). The liberated molecules can signal the recruitment of undifferentiated dental pulp stem cells (DPSCs) to differentiate into odontoblast-like cells and induce dentine repair and regeneration (Galler et al., 2011; Huang et al., 2006; Pang et al., 2014).

Amongst the growth factors identified in the extracellular matrix of dentine, TGF- β 1 is known to play a key role in tooth morphogenesis and odontoblast differentiation (Arany et al., 2014; Begue-Kirn et al., 1992). The dentine matrix bioactive components also play crucial roles in general wound healing and repair processes throughout life (Alaee et al., 2014; Pakyari et al., 2013; Zhang et al., 2011).

Within the dental pulp tissue reside mesenchymal progenitor cells with the ability to differentiate into various mesenchymal cell lineages (Volponi et al., 2010). Multipotent progenitor cells have been isolated from the pulpal tissue of human exfoliated deciduous teeth (SHEDs) (Miura et al., 2003) and permanent teeth (DPSCs) (Gronthos et al., 2000). The DPSCs are believed to reside

within specialized microenvironments called the “stem cell niche”, which are usually quiescent and become activated only after stimulation or injury (Shi & Gronthos, 2003; Waddington et al., 2009; Zhang et al., 2008).

In the presence of mineralization supplements *in vitro*, DPSCs have been reported to differentiate onto odontoblast-like cells and generate mineralized deposits after treatment with dentine matrix extracts (Liu et al., 2005). Furthermore, *in vivo* transplantation of SHEDs and DPSCs within human root dentine in mice, demonstrated the ability of these cells to generate dental tissue in the form of dentine/pulp-like complexes (Huang et al., 2010; Rosa et al., 2013).

Considering this healing and repair capacity of the dentine-pulp complex and the possibility of inducing a growth factor-mediated response by application of materials, clinical protocols can be optimized in order to induce a calcific barrier at the base of deep cavities. This can ultimately help limit injury to a viable dental pulp and, in turn, improve the predictability of vital pulp treatments.

Within the current dental adhesive protocols, the conditioning/etching step applied in conjunction with glass ionomer and composite resin restorations is primarily intended to remove the smear layer and demineralize dentine. This action is necessary for the formation of a hybrid layer for composite resin to adhere to dentine, and to achieve an effective bond between the glass ionomer and dentine (Pashley, 2003; Raggio et al., 2010). However, the ability of these agents in solubilizing and releasing dentine matrix components may provide a mechanism for extending the properties of such materials beyond a mere physicochemical impact for which they had been originally developed (Galler et al., 2015).

There is currently a shortage of information on the biological impact of the components relevant to clinically used conditioning agents on the dentine pulp complex, particularly so with glass ionomers. A previous study (Sadaghiani et al., 2016) reported the failure of polyacrylic acid in solubilizing bioactive proteins from pulverized dentine, which might translate to an inability to induce a biological response from DPSCs.

The current laboratory-based study aims to further investigate the comparative effect of conditioning agents relevant to glass ionomer and composite restorative systems on growth factor release and exposure from dentine, and dentine surface coverage by DPSCs.

It must be noted that we have had to apply exaggerated etching times to achieve growth-factor liberation at quantifiable levels above the limit of detection of the laboratory

methods employed (i.e. TGF- β 1 enzyme-linked immunosorbent assay (ELISA)). It is emphasized that the study does not advocate extending the recommended etching/conditioning time clinically as it impacts the microstructure of dentine, and adversely affects the properties of the bond interface and its resulting strength (Hashimoto et al., 2002).

MATERIALS AND METHODS

Preparation and chemical treatment of dentine slices

Caries-free human third molar and premolar teeth were collected from Oral Surgery clinics, School of Dentistry, Cardiff University, UK, with ethical approval (07/WSE04/84, Wales REC1 and 12WA/0289) and patient consent.

A sharp scalpel (Swann Morton Ltd.) was used to clean the teeth from the attached soft tissue residues before storing in 1% sodium azide (Sigma-Aldrich Ltd.) for disinfection. Teeth were then sectioned horizontally from the occlusal surface towards the cemento-enamel junction into 0.8-1 mm thick slices. Slicing was carried out using a low-speed diamond-edged rotary saw (Testbourne Ltd.) with water coolant. The enamel on the occlusal surface was discarded and dentine slices were used for growth factor quantification (using ELISA and immunogold localization) and cell culture studies. For the cell culture studies, the horizontal slices above the pulp chamber were chosen to ensure a nonperforated surface and even distribution of cells.

The dentine slices were immersed at room temperature in conditioning agents for 5 and 10 min for growth factor quantification experiments, and 5 min for cell culture studies. The conditioning agents were made in the laboratory and included phosphoric acid 37% w/v (pH < 1) (VWR International), citric acid 10% w/v (pH 1.5) (Fisher Scientific), polyacrylic acid 25% w/v (pH 3.9) (VWR International) and EDTA 10% w/v pH adjusted to 7.2 (Fisher Scientific) (positive control here). Phosphate Buffered Saline (PBS) (ThermoFisher Scientific) served as a negative control. Triplicate samples were used per condition for each experiment.

For growth factor quantification, after 5 and 10 min immersion in each solution in separate wells of a 24-well plate (with consistent dentine surface area and weight), the solutions were removed immediately, neutralized to pH 7.0–7.2 using sodium hydroxide and frozen at -20°C for subsequent quantification of growth factors by ELISA. The dentine slices were washed thoroughly in distilled water, dabbed sideways on a sterile towel, and air dried in covered well plates for 48 h prior to immunogold localization of growth factors. Dentine samples were stored in

sealed containers at -20°C prior to cell culture studies. All the experiments and specimen handling were undertaken using anticontamination protocols including the use of gloves, sterile instruments and work surface decontamination with 70% ethanol prior to experiments.

Enzyme-linked immunosorbent assay for TGF- β 1 release

TGF- β 1 was quantified in the solutions using a commercial ELISA development kit (Human/Mouse; eBiosciences). The kit contained capture antibody, biotinylated detection antibody, and avidin HRP. The assay was carried out according to the manufacturer's instructions in triplicates, per condition ($n = 9$). Growth factor concentrations were calculated using a standard curve. Mean values were analysed using the SPSS statistical package.

Immuno-gold localization of growth factors on conditioned dentine sliced surfaces

Conditioned dentine slices were exposed to polyclonal rabbit anti-TGF- β 1 (1:100, Santa Cruz Biotechnology Inc.) primary antibodies and gold-labelled secondary antibodies (1:100, particle size 30 nm, BBI Solutions), followed by silver-enhancing agent (BBI Solutions) prior to carbon coating and visualization using a scanning electron microscope (SEM). Triplicate samples were used and gold-labelled particles of uniform shape and size were counted on 5 random fields of $20\ \mu\text{m}^2$ per slice ($n = 15$), disregarding larger particles of random shape. Mean values were analysed using the SPSS statistical package.

The immunogold localization methodology was validated in advance where detection of gold-labelled particles on dentine surfaces treated by $\text{Ca}(\text{OH})_2$ for 5 min served as a positive control, whilst withdrawal of anti-TGF- β 1 primary antibody prior to incubation with gold-labelled secondary antibodies served as a negative control. An additional negative control was used in which immunolabelling was inhibited by pre-incubation with a 10-fold excess (1:10) of specific blocking peptide (Santa Cruz) diluted in 1% BSA, TBS.

Influence of conditioner on DPSCs morphology and settlement on dentine surface

A dental pulp progenitor population (DPSC) (characterized by Lee et al., 2015) at passage 14 were used

to investigate the impact of dentine conditioning by the above-mentioned agents on DPSC behaviour. The methods for DPSC culture on the dentine surfaces and processing of the specimens for subsequent SEM observation were adapted from methods described by Huang et al. (2006). Cells were cultured on conditioned dentine slices at 3.5×10^4 cells (in 50 μ l volumes) per slice, in 24-well plates in media containing α -MEM, 7% heat deactivated foetal bovine serum, 100 units/ml penicillin G sodium, 0.1 μ g/ml streptomycin sulphate and 0.25 μ g/ml amphotericin (all Gibco, Invitrogen) and 100 μ M L-ascorbate 2-phosphate (Sigma-Aldrich), at 37°C with 5% CO₂. After 2 and 8 days in culture, the slices were washed with PBS and fixed in 2% paraformaldehyde, 2.2% glutaraldehyde in 0.1 M cacodylate buffer, then rinsed in cacodylate buffer, dehydrated in graded ethanol, and air dried at room temperature in fresh 24-well plates prior to carbon coating and SEM analysis.

Statistical analyses

Following the assessment of ELISA and immunogold data through histograms and normal plots (Kolgorov-Smirnov test of normality, $p < .05$), the data was shown to be skewed. Following log transformation, a few outliers remained. Thus, a nonparametric one-way ANOVA (Kruskal-Wallis test) and *post-hoc* Mann-Whitney *U* (MWU) test (adjusted by the Bonferroni correction for multiple tests), was applied on the original data for comparing between groups at each time point. Furthermore, the MWU test for the unpaired data was applied to assess changes for each group with increasing exposure time from 5 to 10 min. All calculations were carried out via SPSS V27 package.

RESULTS

Growth factor release and exposure after dentine conditioning

ELISA detected the release of TGF- β 1 into the conditioning solution with all the agents after 5 min of immersion. The concentration of the growth factor was highest after conditioning with citric acid (MWU with Bonferroni correction, $p < .01$ compared with phosphoric and polyacrylic acid). When increasing the immersion time to 10 min, citric acid, polyacrylic acid and EDTA treatments showed a decrease in the amount of detectable TGF- β 1 (MWU, $p > .05$). Comparatively, conditioning with phosphoric acid resulted in a significant increase in the concentration of the released growth factor over time (MWU, $p < .01$). After 10 min of conditioning, the concentration of TGF- β 1 was significantly lower in the aqueous solution of polyacrylic acid compared with citric acid and phosphoric acid ($p < .01$). As well as this, the aqueous solution of EDTA treatment was demonstrated to contain a significantly lower concentration of growth factor compared with phosphoric acid ($p < .05$) (MWU with Bonferroni correction).

ELISA median values and 95% confidence intervals are presented in Figure 1.

Immunogold labelling revealed that all the conditioning agents exposed TGF- β 1 on dentine surfaces. After 5 min of treatment, citric acid was the most effective agent in exposing the growth factor on dentine surface, while phosphoric acid was significantly less effective than other agents (MWU with Bonferroni correction, $p < .05$). An increase in dentine surface levels of TGF- β 1 was observed between 5 and 10 min with phosphoric acid conditioning, while conditioning with all the other

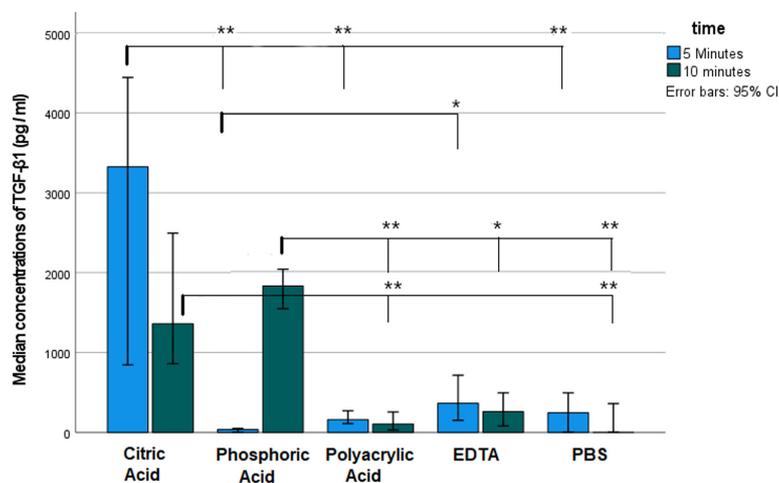


FIGURE 1 Median and 95% CI for TGF- β 1 concentration at 5 and 10 min as measures by ELISA. ** and * represent significant differences between the marked test groups at $p < .01$ and $p < .05$ respectively; MWU test with Bonferroni correction.

agents resulted in a drop in TGF- β 1 dentine surface level over time (MWU, $p < .01$ for all). After 10 min of conditioning, dentine surfaces treated with phosphoric acid displayed the highest level of TGF- β 1 compared to citric acid and polyacrylic acid (MWU with Bonferroni correction, $p < .05$ and $.01$ respectively). Immunogold labelling median values and 95% confidence intervals are presented in Figure 2.

Figure 3 shows representative SEM images of conditioned dentine slices after gold labelling for TGF- β 1.

FIGURE 2 Median and 95% CI for TGF- β 1 counts of immunogold-labelled particles per $20\ \mu\text{m}^2$ at 5 and 10 min. ** and * represent significant differences between the marked test groups at $p < .01$ and $p < .05$ respectively; MWU test with Bonferroni correction.

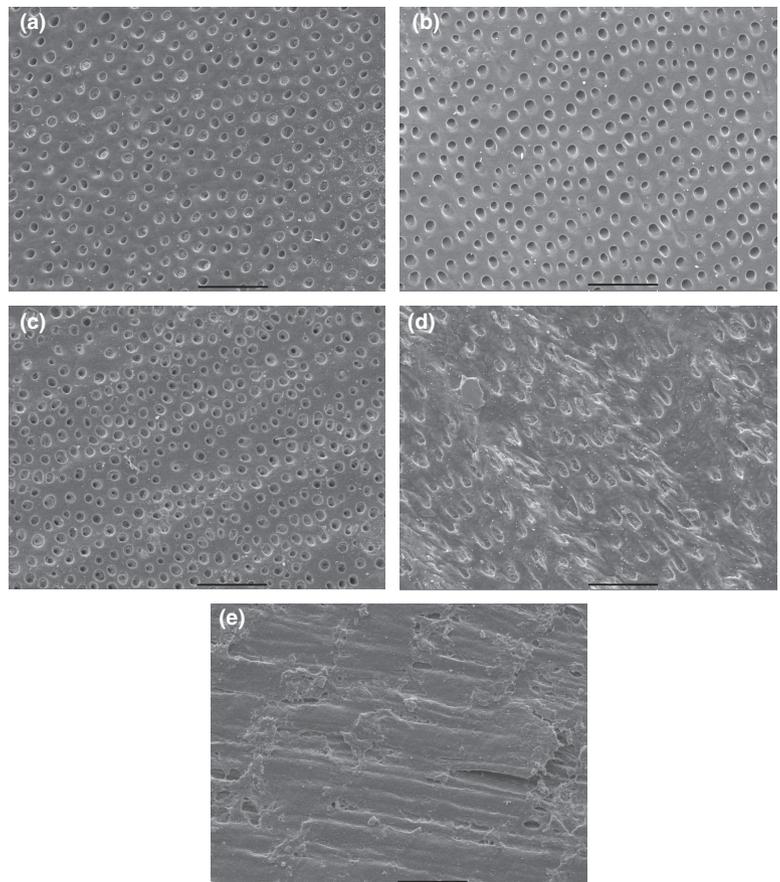
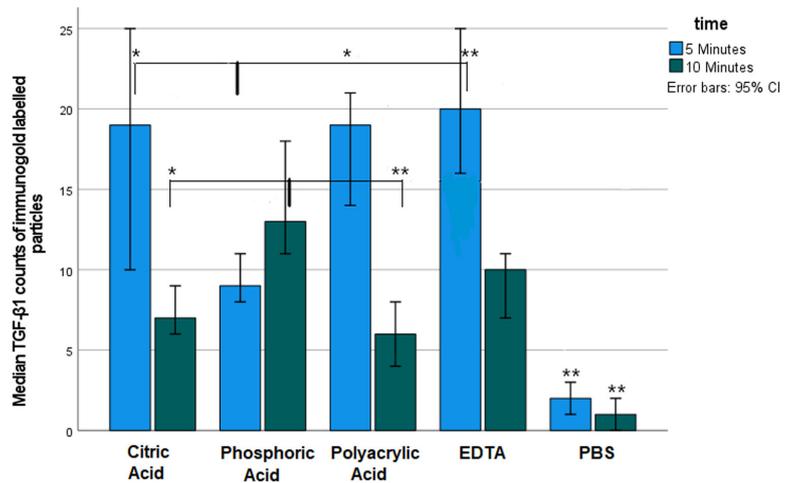


FIGURE 3 Immunogold labelling of TGF- β 1 on dentine surfaces (treated for 5 min with: (a) citric acid, (b) phosphoric acid, (c) polyacrylic acid, (d) ethylenediaminetetraacetic acid (EDTA), and (e) phosphate buffered saline (PBS). The smear layer is intact on the surface exposed to PBS masking the dentinal tubules (scale bar shows $20\ \mu\text{m}$).

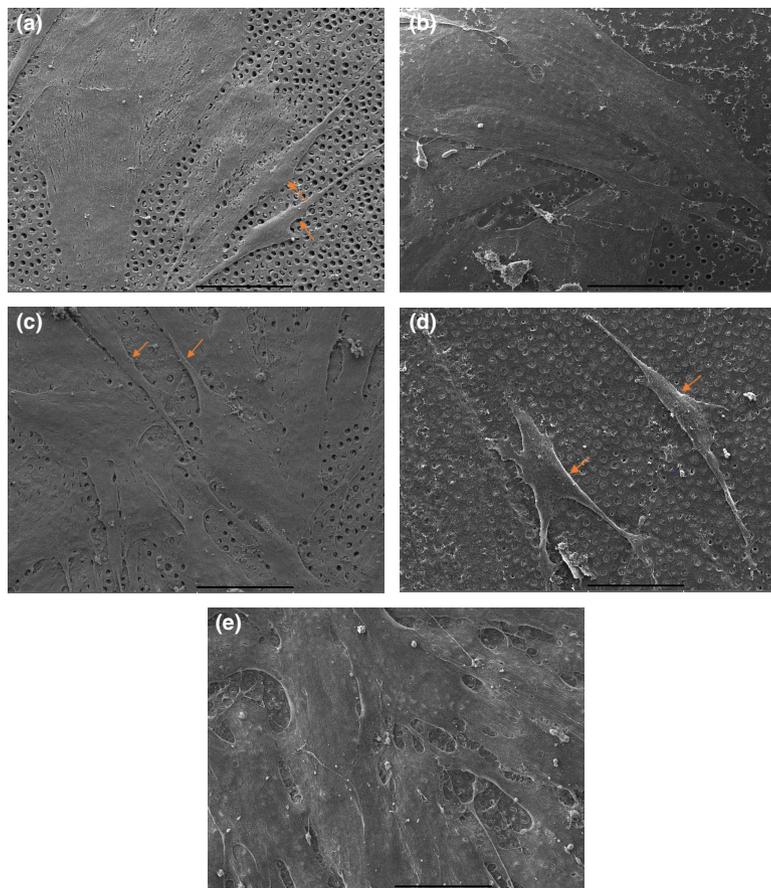


FIGURE 4 Representative SEM images on day 2 showing comparable DPSCs coverage against the underlying dentinal tubules on dentine surfaces treated with: (a) citric acid, (b) phosphoric acid, (c) polyacrylic acid, and (d) ethylenediaminetetraacetic acid (EDTA). In some areas, prominent individual cell bodies and their processes can be identified (arrows), while the outline of cells in the remaining areas appears spread and overlapped. (e) The cell outlines are not clear against the smear layer-clogged PBS-treated dentine (magnification 1.4 k- scale bar shows 50 μm).

possible due to overlapping cell bodies in some areas. It was not possible to make out the cell outlines against the smear layer-clogged surface of PBS-treated dentine (Figure 4).

On day 8, the visual inspection of the SEM images showed denser cell coverage on citric acid and EDTA-treated dentine compared with day 2. The cell coverage on polyacrylic acid-treated dentine was patchy compared to the abovementioned specimens where the cells showed almost complete coverage of dentine surface. The cell coverage on phosphoric acid-treated dentine although widespread, appeared different to that of citric acid and EDTA-treated dentine in that the cell boundaries were identifiable and raised compared to the overlapped and spread outline of cells on the latter (Figure 5).

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. The exposure and release of TGF- β 1 from dentine was investigated as this is known to be an important growth factor for inducing mineralized phenotype differentiation of dental stem cells (Arany et al., 2014). For conditioning dentine slices we used

phosphoric acid, representing a strong etching agent used clinically in conjunction with resin composite restorative systems, citric acid, representing a weaker organic acidic dentine conditioner, and polyacrylic acid, a conditioning agent and constituent of glass ionomer (GI) systems. EDTA, a neutral chelating agent, used for smear layer removal clinically, served as positive control here.

The indirect effect on DPSCs by the applied agents mediated through the released bioactive molecules from dentine has been proposed as an important mechanism for inducing a dentinogenic response in the pulp (Smith et al., 2001; Smith et al., 2012). This effect is particularly important when restoring deep cavities in teeth with viable dental pulps. In such situations, clinicians face a dilemma whether to directly ‘bond’ a composite restoration or to place a ‘base’ first using glass-ionomer cement (the sandwich technique). There are reports of an inconsistent range of approaches when managing this clinical situation by dental practitioners and within clinical teaching environments (Gilmour et al., 2009; Wilson & Lynch, 2014). The evidence is currently inconclusive as to the comparative superiority of each approach relevant to microleakage control potential (Gungor et al., 2014; Kasraei et al., 2011; Moazzami et al., 2014) and is almost lacking relating to the potential differences in terms of the biological impact on the pulp.

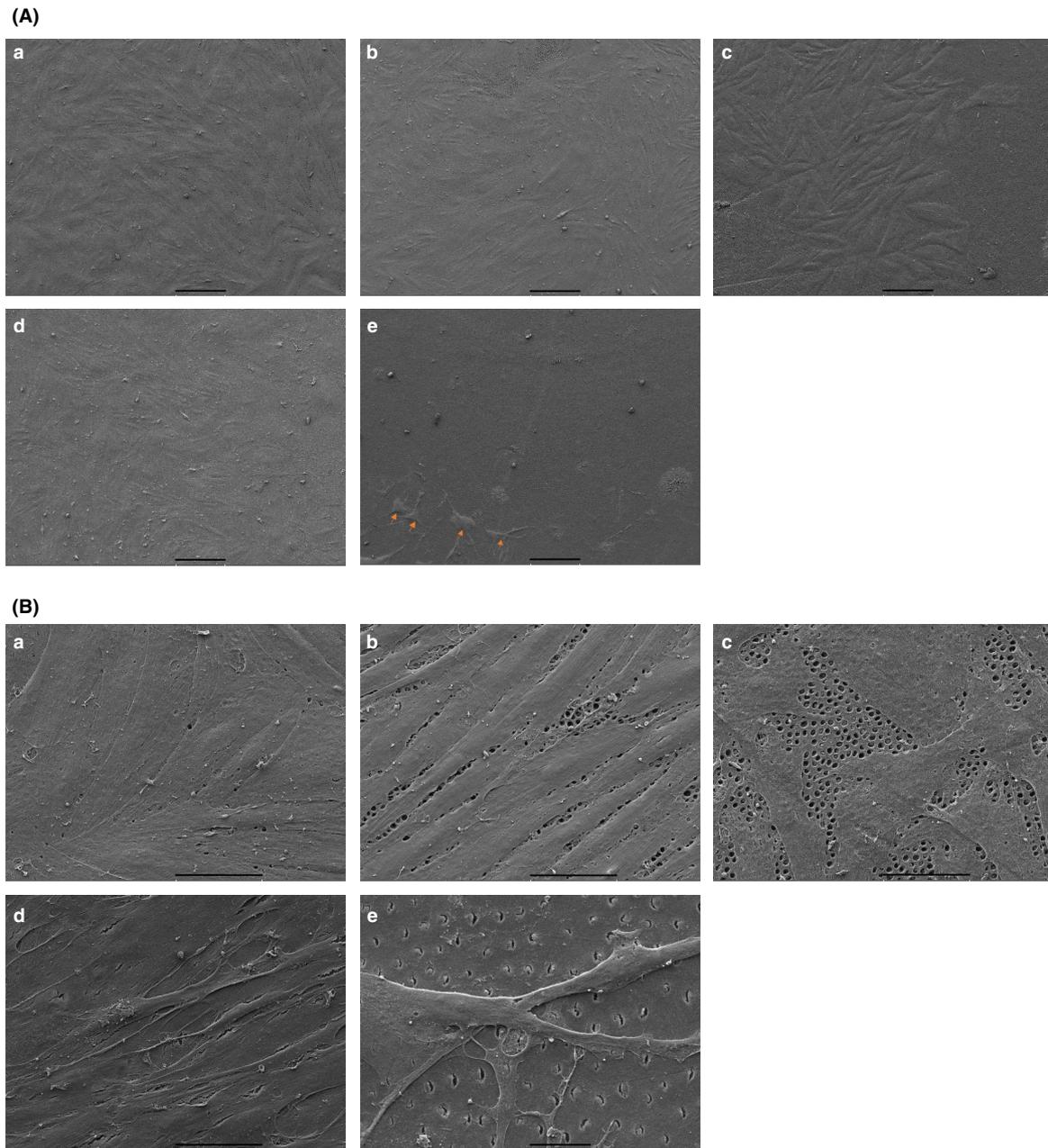


FIGURE 5 (A) Representative SEM images on day 8 at magnification $\times 200$ showing comparative cell coverage on dentine surfaces treated with: (a) citric acid, (b) phosphoric acid, (c) polyacrylic acid (note areas devoid of cells with dentinal tubules visible), (d) EDTA, and (e) PBS. On the PBS-treated dentine surface, only several cells marked by arrows are seen in the lower border of the image in contrast to the other groups where uniform cell coverage makes it impossible to identify individual cell outlines at this magnification (scale bar shows $200\ \mu\text{m}$) (B) Note the individual outline of cells on (b) phosphoric acid-treated dentine and patchy cell coverage on (c) polyacrylic acid-treated dentine, compared to the spread and overlapped cell outlines on citric acid and EDTA-treated dentine ((a) to (d) magnification $\times 1.4\ \text{k}$, (bar shows $50\ \mu\text{m}$). Smear layer residue is present on PBS-treated dentine (e) clogging the dentinal tubules; an isolated cell and its cellular processes are shown at magnification $\times 2.4\ \text{k}$ (scale bar shows $20\ \mu\text{m}$).

Findings of our previous study (Sadaghiani et al., 2016) showed differences in the ability of etching/conditioning agents in isolating dentine matrix proteins from pulverized dentine. Importantly, the failure of polyacrylic acid in extracting detectable proteins from dentine powder suggested that potential differences in terms of inducing

a biological response may exist between the two clinical approaches.

In the present study following conditioning of dentine for 5 min, the highest concentration of TGF- $\beta 1$ was detected in the aqueous solution of citric acid conditioning. When conditioning dentine with phosphoric and

polyacrylic acids for the same period, significantly less concentrations of the growth factor were detected by ELISA. This may be explained by the relative weakness of polyacrylic acid and its ineffectiveness in growth factor release, and the harsh nature of phosphoric acid potentially denaturing the released growth factors (O'Brien et al., 2012). A key point to emphasize is that the conditioning times applied here are significantly longer than those advocated for clinical applications. Prolonged application of acidic agents clinically can result in enamel and dentine erosion and can have detrimental effects on the bond strength of the restoration (Hashimoto et al., 2002; West et al., 2000). However, to enable the detection and comparison of growth factor liberation, we applied exaggerated conditions in this laboratory study starting at a baseline conditioning time of 5 min. Whilst it is acknowledged that dentine is conditioned for significantly shorter times in a clinical setting, by further extending the conditioning time to 10 min we aimed to investigate the kinetics of growth factor release over time.

After 10 min of conditioning of dentine with phosphoric acid, significant increase in growth factor concentration in the solution was detected. This may be explained by the ultra-low pH (<1) of the acid, causing demineralisation of dentine and structural breakdown of the matrix over the extended time (Breschi et al. 2003; West et al., 2000), releasing a significantly higher volume of proteins from the deeper layers. Comparatively, a reduction in growth factor concentration was detected when extending conditioning time with the other conditioning agent. This may be due to denaturing of the growth factor previously released into the solution.

In line with methods described by Zhao et al. (2000), immunogold labelling was employed to mark TGF- β 1 exposure on the surface. Apart from the round particles of uniform size, sporadic larger particles of random shape could also be observed under SEM. Although studies on immunogold localization of dentine matrix components are sparse, similar observations were made by Lin et al. (1993) concerning immunolocalization of collagen type I on EDTA-treated dentine surfaces. Using $\times 100\,000$ magnification, the authors explained the larger particles of random shape were likely residual minerals on the collagen fibrils and could be differentiated from the immunogold particles through their irregular shape and larger size. Accordingly, the current study excluded those particles from the immunogold counts.

Similar to the Zhao et al. (2000) findings, a small number of gold-labelled particles on dentine specimens treated with PBS was detected. These labelled particles mark the growth factor in the smear layer residue, as evident on the SEM images. Bearing in mind the smear layer was removed after conditioning by the acidic and chelating

agents (as evident by the apparently patent dentinal tubules), it is expected that the immunolabelled particles on the later specimens mark the surface growth factor attached to the dentine matrix.

The surface detection of TGF- β 1 was significantly lower in phosphoric acid-treated specimens compared with all the remaining groups after a 5-min conditioning. The comparative higher surface levels of the growth factor in the later groups are possibly due to the cleavage of growth factor from other proteins of dentine matrix, unravelling them and facilitating their early detection, followed by denaturation/conformative changes of the molecules in the extended conditioning time (O'Brien et al., 2012). With phosphoric acid, the lower surface levels of TGF- β 1 in the earlier time point are likely due to a comparatively earlier breakdown and denaturation of the surface growth factors. Deeper demineralization associated with the ultralow pH of phosphoric acid and breakdown of the dentine matrix in extended exposure time (Breschi et al. 2003) can result in renewing the surface growth factor resulting in an increase in detection of TGF- β 1 after 10 min. Although the conditioning times applied in this *in vitro* study are far longer than those advocated for clinical application, the results provide evidence on the potential detrimental effect of prolonged dentine conditioning by strong acids from a biological standpoint.

The potential of the conditioned dentine surfaces in supporting DPSCs in culture was investigated by SEM imaging at the early stage of day 2 and later at day 8. As cell settlement (and attachment) to dentine takes place prior to proliferation and differentiation to odontoblast-like cells (Pang et al., 2014; Ring et al., 2008), coverage of dentine surface by DPSCs can be considered an important initial step in the process. The observed elongated and stretched cell morphology has been attributed to odontoblast-like cells (Huang et al., 2006), however, it is acknowledged that for confirming the cell lineage, specific gene-expression experiments would be needed. No striking differences between the groups in terms of cell coverage were evident by visual inspection on day 2; the coverage was incomplete for all groups at this stage, with some individual cell outlines identifiable. On day 8 however, the apparent widespread cell coverage and overlapping of the cells cultured on EDTA and citric acid-treated dentine suggested the supportiveness of the surfaces for DPSCs. On the phosphoric acid-treated dentine, the cell bodies did not appear as spread and overlapped. This may indicate a weaker attachment of cells on phosphoric acid-treated dentine. However, to support this assumption, further investigation such as the assessment of cell adhesion proteins over an extended period in culture is required. The cell coverage on polyacrylic acid-treated dentine was comparatively sparse,

indicating a surface less attractive and less supportive to the cells. Considering these findings alongside growth factor release and exposure data, an association is observed between the growth factor presence in the culture environment and cell coverage of dentine surfaces.

Our previous study (Sadaghiani et al., 2016) suggested that while liberation of growth factors from dentine apparently correlates with a dentinogenic response of DPSCs *in vitro*, other important mechanisms may also be involved in an overall favourable response towards dentine regeneration. The effect of conditioning on dentine surface topography and surface hardness may be an additional determining factor. Several studies have demonstrated the impact of surface topography (i.e. roughness) on stem cell behaviour. It has been shown that the nanotopographical pattern of a biomaterial on which stem cells are cultured may influence adhesion, proliferation and differentiation of cells to osteogenic lineage (Dalby et al., 2007; Kolind et al., 2014; Oh et al., 2009). These studies showed a weak cellular adhesion and differentiation in association with highly ordered topographies, and emphasized the importance of substrate physical characteristics in cell fate. In terms of the biological mechanisms involved, it has been proposed that the effect of topography on cells may be exerted by altering integrin clustering, focal adhesion assemblies, cytoskeletal organization, and nuclear mechanosensing which may ultimately result in adaptive gene and protein-level changes in response to biophysical signals from the extracellular microenvironment (Hamilton & Brunette, 2007; Kokubu et al., 2009; Li & Xie, 2007; Teo et al., 2010; Yim et al., 2010). Furthermore, prolonged acid etching with strong acids has been shown to cause deep demineralisation of dentine, rendering the surface almost devoid of minerals (Hashimoto et al., 2002; Wang and Spencer 2004), and ultimately affecting the hardness (Angker et al. 2004). Stiffness of the surface is shown to influence differentiation of stem cells cultured on it (Fu et al. 2010), providing another potential reason for differences observed in cell behaviour related to different conditioning agents used here.

In line with the findings reported in other studies, our observation in a previous study (Alshumrani A, MSc dissertation, Cardiff University) showed treatment for 5 min by the agents used here, caused an increase in surface roughness, and decrease in hardness of polished dentine slices. Polyacrylic acid-treated dentine had the least increase in roughness and decrease in hardness after conditioning. The mechanical characteristics of dentine treated with polyacrylic acid, combined with its inferior ability in dentine growth factor liberation can provide an explanation for a surface relatively unattractive for DPSCs.

Finally, the direct effect of the conditioning agents on the cells should not be overlooked. Although dentine

slices were washed copiously in distilled water after conditioning here, any residue of the conditioning agent could have an adverse impact on cell survival; culturing rat pulp cells in the presence of 100 times dilution of phosphoric acid (to 0.37%) was shown to cause marked decrease in cell viability compared with another diluted etching agent (0.1% phytic acid) *in vitro* (Nassar et al., 2013).

This study revealed a less favourable cellular response, in terms of DPSC settlement and coverage, on dentine surfaces treated by polyacrylic, and phosphoric acid-treated dentine compared with that of EDTA and citric acid. Quantification of the liberated TFG- β 1 suggest a potential growth factor-mediated response influencing cell behaviour. As for the implications of our findings for clinical practice, polyacrylic acid as a key constituent of the glass ionomer system, does not appear to evoke a desirable biological response from DPSCs. With the apparent desirable biological outcome related to dentine conditioning with citric acid and EDTA observed here, considerations should be given to replacing phosphoric acid by milder acids as the etching agent in adhesive restorative systems. The 10% citric acid and 17% EDTA have been previously shown to be capable of etching dentine, and to be effective in removing the smear layer, smear plugs, demineralizing intertubular dentine, and exposing a stable collagen matrix (Bogra and Kaswan 2003; Breschi et al., 2002).

Concerns have been expressed regarding the composite to enamel bond strength in the self-etch adhesive systems, attributed to the lower etching capacity of milder acids in these systems, compared with the etch-and-rinse systems where phosphoric acid is used as the etching agent (Brackett et al., 2006; Ernest et al., 2004; Goracci et al., 2004). As well as this, the possible long-term detrimental effects of matrix metalloproteinases on dentine-composite bond interface, released from dentine following dentine etching by stronger acidic agents have also been raised (Imbery et al. 2012). Considering these inconsistent perspectives, optimization of the current tooth-conditioning protocols is important not only for achieving a more reliable and durable bond strength, but also to induce a desirable biological response in teeth with vital pulps.

CONCLUSION

Based on the findings of this *in vitro* study, a desirable biological growth factor-mediated effect may be gained when conditioning dentine with milder acidic or chelating agents such as citric acid and EDTA. The results must be interpreted in the context that the potential of the applied materials in inducing a desirable biological response in DPSCs, is only one consideration amongst other important ones in a clinical setting. However, it is crucial to look

beyond the mere physical effects of materials and move towards biologically based treatment approaches as far as the restorative management of teeth with viable dental pulps is concerned.

AUTHOR CONTRIBUTIONS

Study conception and design: L Sadaghiani, AJ Sloan. Acquisition of data: L Sadaghiani, Gleeson HB, AM Alshumrani. Analysis and interpretation of data: L Sadaghiani, HB Gleeson, AM Alshumrani, W Nishio Ayre, AJ Sloan. Drafting of manuscript: L Sadaghiani. Critical revision: L Sadaghiani, HB Gleeson, AM Alshumrani, W Nishio Ayre, AJ Sloan.

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available.

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REFERENCES

- Alaee, F., Hong, S.H., Dukas, A.G., Pensak, M.J., Rowe, D.W. & Lieberman, J.R. (2014) Evaluation of osteogenic cell differentiation in response to bone morphogenetic protein or demineralized bone matrix in a critical sized defect model using GFP reporter mice. *Journal of Orthopaedic Research*, 32, 1120–1128.
- Angker, L., Nockolds, C., Swain, M.V. & Kilpatrick, N. (2004) Correlating the mechanical properties to the mineral content of carious dentine--a comparative study using an ultra-micro indentation system (UMIS) and SEM-BSE signals. *Archives of Oral Biology*, 49, 369–378.
- Arany, P.R., Cho, A., Hunt, T.D., Sidhu, G., Shin, K., Hahm, E. et al. (2014) Photoactivation of endogenous latent transforming growth factor-beta1 directs dental stem cell differentiation for regeneration. *Science Translational Medicine*, 6, 238ra269.
- Baker, S.M., Sugars, R.V., Wendel, M., Smith, A.J., Waddington, R.J., Cooper, P.R. et al. (2009) TGF-beta/extracellular matrix interactions in dentin matrix: a role in regulating sequestration and protection of bioactivity. *Calcified Tissue International*, 85, 66–74.
- Begue-Kirn, C., Smith, A.J., Ruch, J.V., Ruch, J.V., Wozney, J.M., Purchio, A. et al. (1992) Effects of dentin proteins, transforming growth factor beta 1 (TGF beta 1) and bone morphogenetic protein 2 (BMP2) on the differentiation of odontoblast in vitro. *The International Journal of Developmental Biology*, 36, 491–503.
- Bogra, P. & Kaswan, S. (2003) Etching with EDTA- an in vitro study. *Journal of the Indian Society of Pedodontics and Preventative Dentistry*, 21, 79–83.
- Brackett, W.W., Ito, S., Nishitani, Y., Haisch, L.E. & Pashley, D.H. (2006) The microtensile bond strength of self-etching adhesives to ground enamel. *Operative Dentistry*, 31, 332–337.
- Breschi, L., Gobbi, P., Mazzotti, G., Falconi, M., Ellis, T.H. & Stangel, I. (2002) High resolution SEM evaluation of dentin etched with maleic and citric acid. *Dental Materials*, 18, 26–35.
- Breschi, L., Perdigão, J., Gobbi, P., Mazzotti, G., Falconi, M. & Lopes, M. (2003) Immunocytochemical identification of type I collagen in acid-etched dentin. *Journal of Biomedical Materials Research*, 66, 764–769.
- Dalby, M.J., Gadegaard, N., Tare, R., Andar, A., Riehle, M.O., Herzyk, P. et al. (2007) The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nature Materials*, 6, 997–1003.
- Ernest, C.P., Holzmeier, M. & Willershausen, B. (2004) In vitro shear bond strength of self-etching adhesives in comparison to 4th and 5th generation adhesives. *Journal of Adhesive Dentistry*, 6, 293–299.
- Ferracane, J.L., Cooper, P.R. & Smith, A.J. (2013) Dentin matrix component solubilization by solutions at pH relevant to self-etching dental adhesives. *The Journal of Adhesive Dentistry*, 15, 407–412.
- Fu, J., Wang, Y.K., Yang, M.T., Desai, R.A., Yu, X., Liu, Z. et al. (2010) Mechanical regulation of cell function with geometrically modulated elastomeric substrates. *Nature Methods*, 7, 733–736.
- Galler, K.M., Buchalla, W., Hiller, K.A., Federlin, M., Eidt, A., Schiefersteiner, M. et al. (2015) Influence of root canal disinfectants on growth factor release from dentin. *Journal of Endodontics*, 41, 363–368.
- Galler, K.M., D'Souza, R.N., Federlin, M., Cavender, A.C., Hartgerink, J.D., Hecker, S. et al. (2011) Dentin conditioning codetermines cell fate in regenerative endodontics. *Journal of Endodontics*, 37, 1536–1541.
- Gilmour, A.S., Latif, M., Addy, L.D. & Lynch, C.D. (2009) Placement of posterior composite restorations in United Kingdom dental practices: techniques, problems, and attitudes. *International Dental Journal*, 59, 148–154.
- Goracci, C., Sadek, F.T., Monticelli, F., Cardoso, P.E. & Ferrari, M. (2004) Microtensile bond strength of self-etching adhesives to enamel and dentin. *Journal of Adhesive Dentistry*, 6, 313–318.
- Graham, L., Cooper, P.R., Cassidy, N., Nor, J.E., Sloan, A.J. & Smith, A.J. (2006) The effect of calcium hydroxide on solubilisation of bio-active dentine matrix components. *Biomaterials*, 27, 2865–2873.
- Gronthos, S., Mankani, M., Brahimi, J., Robey, P.G. & Shi, S. (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, 97, 13625–13630.
- Gungor, H.C., Canoğlu, E. & Cehreli, Z.C. (2014) The effects of dentin adhesives and liner materials on the microleakage of class II resin composite restorations in primary and permanent teeth. *Journal of Clinical Paediatric Dentistry*, 38, 223–228.
- Hamilton, D.W. & Brunette, D.M. (2007) The effect of substratum topography on osteoblast adhesion mediated

- signal transduction and phosphorylation. *Biomaterials*, 28, 1806–1819.
- Hashimoto, M., Ohno, H., Kaga, M., Sano, H., Tay, F.R., Oguchi, H. et al. (2002) Over-etching effects on micro-tensile bond strength and failure patterns for two dentin bonding systems. *Journal of Dentistry*, 30, 99–105.
- Huang, G.T., Shagranova, K. & Chan, S.W. (2006) Formation of odontoblast-like cells from cultured human dental pulp cells on dentin in vitro. *Journal of Endodontics*, 32, 1066–1073.
- Huang, G.T., Yamaza, T., Shea, L.D., Djouad, F., Kuhn, N.Z., Tuan, R.S. et al. (2010) Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model. *Tissue Engineering Part A*, 16, 605–615.
- Imbery, T.A., Kennedy, M., Janus, C. & Moon, P.C. (2012) Evaluating EDTA as a substitute for phosphoric acid-etching of enamel and dentin. *General Dentistry*, 60, 55–61.
- Kasraei, S., Azarsina, M. & Majidi, S. (2011) In vitro comparison of microleakage of posterior resin composites with and without liner using two-step etch-and-rinse and self-etch dentin adhesive systems. *Operative Dentistry*, 36, 213–221.
- Kokubu, E., Hamilton, D.W., Inoue, T. & Brunette, D.M. (2009) Modulation of human gingival fibroblast adhesion, morphology, tyrosine phosphorylation, and ERK 1/2 localization on polished, grooved and SLA substratum topographies. *Journal of Biomedical Materials Research Part A*, 91, 663–670.
- Kolind, K., Kraft, D., Bøggild, T., Duch, M., Lovmand, J., Pedersen, F.S. et al. (2014) Control of proliferation and osteogenic differentiation of human dental-pulp-derived stem cells by distinct surface structures. *Acta Biomaterialia*, 10, 641–650.
- Lee, C.P., Colombo, J.S., Ayre, W.N., Sloan, A.J. & Waddington, R.J. (2015) Elucidating the cellular actions of demineralised dentine matrix extract on a clonal dental pulp stem cell population in orchestrating dental tissue repair. *Journal of Tissue Engineering*, 14, 2041731415586318.
- Li, J.J. & Xie, D. (2007) Cleavage of focal adhesion kinase (FAK) is essential in adipocyte differentiation. *Biochemical and Biophysical Research Communications*, 357, 648–654.
- Lin, C.P., Douglas, W.H. & Erlandsen, S.L. (1993) Scanning electron microscopy of type I collagen at the dentin-enamel junction of human teeth. *The Journal of Histochemistry and Cytochemistry*, 41, 381–388.
- Liu, J., Jin, T., Ritchie, H.H., Smith, A.J. & Clarkson, B.H. (2005) In vitro differentiation and mineralization of human dental pulp cells induced by dentin extract. *In Vitro Cellular & Developmental Biology/Animal*, 41, 232–238.
- Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, L.W., Robey, P.G. et al. (2003) SHED: stem cells from human exfoliated deciduous teeth. *Proceedings of the National Academy of Sciences of the United States of America*, 100, 5807–5812.
- Moazzami, S.M., Sarabi, N., Hajizadeh, H., Majidinia, S., Li, Y., Meharry, M.R. et al. (2014) Efficacy of four lining materials in sandwich technique to reduce microleakage in class II composite resin restorations. *Operative Dentistry*, 39, 256–263.
- Nassar, M., Hiraishi, N., Islam, M.S., Aizawa, M., Tamura, Y., Otsuki, M. et al. (2013) Effect of phytic acid used as etchant on bond strength, smear layer, and pulpal cells. *European Journal of Oral Sciences*, 121, 482–487.
- O'Brien, E.P., Brooks, B.R. & Thirumalia, D. (2012) Effects of pH on proteins: predictions for ensemble and single-molecule pulling experiments. *Journal of the American Chemical Society*, 134, 979–987.
- Oh, S., Brammer, K.S., Li, Y.S., Teng, D., Engler, A.J., Chien, S. et al. (2009) Stem cell fate dictated solely by altered nanotube dimension. *Proceedings of the National Academy of Sciences of the United States of America*, 106, 2130–2135.
- Pakyari, M., Farrokhi, A., Maharlooei, M.K. & Ghahary, A. (2013) Critical role of transforming growth factor Beta in different phases of wound healing. *Advances in Wound Care*, 2, 215–224.
- Pang, N.S., Lee, S.J., Kim, E., Shin, D.M., Cho, S.W., Park, W. et al. (2014) Effect of EDTA on attachment and differentiation of dental pulp stem cells. *Journal of Endodontics*, 40, 811–817.
- Pashley, D.H. (2003) The evolution of dentin bonding. *Dentistry Today*, 22, 112–114, 116, 118–119.
- Raggio, D.P., Sónego, F.G., Camargo, L.B., Marquezan, M. & Imparato, J.C. (2010) Efficiency of different polyacrylic acid concentrations on the smear layer, after ART technique, by scanning electron microscopy (SEM). *European Archives of Paediatric Dentistry*, 11, 232–235.
- Ring, K.C., Murray, P.E., Namerow, K.N., Kuttler, S. & Garcia-Godoy, F. (2008) The comparison of the effect of endodontic irrigation on cell adherence to root canal dentin. *Journal of Endodontics*, 34, 1474–1479.
- Rosa, V., Zhang, Z., Grande, R.H.M. & Nör, J.E. (2013) Dental pulp tissue engineering in full-length human root canals. *Journal of Dental Research*, 92, 970–975.
- Sadaghiani, L., Gleeson, H.B., Youde, S., Waddington, R.J., Lynch, C.D. & Sloan, A.J. (2016) Growth factor liberation and DPSC response following dentine conditioning. *Journal of Dental Research*, 95, 1298–1307.
- Shi, S. & Gronthos, S. (2003) Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *Journal of Bone and Mineral Research*, 18, 696–704.
- Sloan, A.J., Moseley, R., Dobie, K., Waddington, R.J. & Smith, A.J. (2002) TGF-beta latency-associated peptides (LAPs) in human dentin matrix and pulp. *Connective Tissue Research*, 43, 381–386.
- Sloan, A.J., Perry, H., Matthews, J.B. & Smith, A.J. (2000) Transforming growth factor-beta isoform expression in mature human healthy and carious molar teeth. *Histochemical Journal*, 32, 247–252.
- Smith, A.J. (2003) Vitality of the dentin-pulp complex in health and disease: growth factors as key mediators. *Journal of Dental Education*, 67, 678–689.
- Smith, A.J., Matthews, J.B. & Hall, R.C. (1998) Transforming growth factor-beta1 (TGF-beta1) in dentine matrix. Ligand activation and receptor expression. *European Journal of Oral Sciences*, 106(Suppl 1), 179–184.
- Smith, A.J., Murray, P.E., Sloan, A.J., Matthews, J.B. & Zhao, S. (2001) Trans-dentinal stimulation of tertiary dentinogenesis. *Advances in Dental Research*, 15, 51–54.
- Smith, J.G., Smith, A.J., Shelton, R.M. & Cooper, P.R. (2012) Recruitment of dental pulp cells by dentine and pulp extracellular matrix components. *Experimental Cell Research*, 318, 2397–2406.
- Teo, B.K., Ankam, S., Chan, L.Y. & Yim, E.K. (2010) Nanotopography/mechanical induction of stem-cell differentiation. *Methods in Cell Biology*, 98, 241–294.
- Tomson, P.L., Grover, L.M., Lumley, P.J., Sloan, A.J., Smith, A.J. & Cooper, P.R. (2007) Dissolution of bio-active dentine matrix

- components by mineral trioxide aggregate. *Journal of Dentistry*, 35, 636–642.
- Van Meerbeek, B., Yoshihara, K., Van Landuyt, K., Yoshida, Y. & Peumans, M. (2020) From Buonocore's pioneering acid-etch technique to self-adhering restoratives. A status perspective of rapidly advancing dental adhesive technology. *The Journal of Adhesive Dentistry*, 22, 7–34.
- Volponi, A.A., Pang, Y. & Sharpe, P. (2010) Stem cell-based biological tooth repair and regeneration. *Trends in Cell Biology*, 20, 715–722.
- Waddington, R.J., Youde, S.J., Lee, C.P. & Sloan, A.J. (2009) Isolation of distinct progenitor stem cell populations from dental pulp. *Cells, Tissues, Organs*, 189, 268–274.
- Wang, Y. & Spencer, P. (2004) Effect of acid etching time and technique on interfacial characteristics of the adhesive-dentin bond using differential staining. *European Journal of Oral Sciences*, 112, 293–299.
- West, N.X., Hughes, J.A. & Addy, M. (2000) Erosion of dentine and enamel in vitro by dietary acids: the effect of temperature, acid character, concentration and exposure time. *Journal of Oral Rehabilitation*, 27, 875–880.
- Wilson, N.H. & Lynch, C.D. (2014) The teaching of posterior resin composites: planning for the future based on 25 years of research. *Journal of Dentistry*, 42, 503–516.
- Yim, E.K., Darling, E.M., Kulangara, K., Guilak, F. & Leong, K.W. (2010) Nanotopography-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of human mesenchymal stem cells. *Biomaterials*, 31, 1299–1306.
- Zhang, C., Chang, J., Sonoyama, W., Shi, S. & Wang, C.Y. (2008) Inhibition of human dental pulp stem cell differentiation by notch signaling. *Journal of Dental Research*, 87, 250–255.
- Zhang, R., Cooper, P.R., Smith, G., Nör, J.E. & Smith, A.J. (2011) Angiogenic activity of dentin matrix components. *Journal of Endodontics*, 37, 26–30.
- Zhao, S., Sloan, A.J., Murray, P.E., Lumley, P.J. & Smith, A.J. (2000) Ultrastructural localisation of TGF-beta exposure in dentine by chemical treatment. *Histochemistry Journal*, 32, 489–494.

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