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Growth factor release from dentine matrix by pulp capping agents promote pulp tissue repair-associated events

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Abstract

Aim To characterise growth factor release from dentine by pulp capping agents and to determine the effects of liberated dentine extracellular matrix (dECM) components on pulp cells in the key wound healing processes of migration and cell growth.

Methodology Powdered human dentine was exposed to solutions of calcium hydroxide, white and grey Mineral Trioxide Aggregate (MTA) (ProRoot, (Dentsply Tulsa, Tulsa, OK, USA) over 14 days. The solubilised dECM components were dialysed and lyophilised and characterised using multiplex quantitative ELISA. Following dECM component extraction dentine was analysed using Fourier transform infrared spectroscopy (FTIR). Primary rat dental pulp cells (RDPCs) were exposed to dECM components (0.1 - 100 µg/mL) released by calcium hydroxide, white and grey MTA and cell growth and chemotactic responses were assessed. Statistical differences between the experimental and control groups were determined using one way ANOVA

Results A broad range of growth factors, many not previously reported in dentine, were liberated by these pulp capping agents, including: SCF, M-CSF, GM-CSF, IGFBP-1, NGF, GDNF. White and grey MTA liberated more growth factors than calcium hydroxide. FTIR analysis of dentine exposed to pulp capping agents showed partial depletion of amide bands I, II and III, with little alteration in phosphate peaks compared to untreated dentine. dECM components released by white and grey MTA induced significantly more cell growth at low-to-moderate concentrations ($p \leq 0.05$) examined in this study, and significantly enhanced cell chemotaxis at all concentrations compared with controls ($p \leq 0.05$).

Conclusions White and grey MTA solubilise a broad range of bioactive molecules from dentine, which can induce proliferation and chemotaxis in pulp cells.

Introduction

Although the compositions of bone and dentine matrix are similar, the absence of physiological extracellular matrix turnover in dentine gives the illusion that dentine is a relatively inert tissue. During primary dentinogenesis growth factors and bioactive molecules are secreted by odontoblasts and which are incorporated within the dentine extracellular matrix (dECM) (Smith & Lesot 2001). These molecules are bound to protein precursors or binding proteins and sequestered in a protected state within dentine (Smith *et al.* 1998, Sloan *et al.* 2002, Baker *et al.* 2009). This matrix-binding provides a robust mechanism for the protection of these bioactive molecules and provides an elegant system for signalling regeneration (Smith *et al.* 2012) when they are released by bacterial acids (Dung *et al.* 1995) during carious attack or by placement of pulp-capping agents such as calcium hydroxide or Mineral Trioxide Aggregate (MTA) (Graham *et al.* 2006, Tomson *et al.* 2007).

Growth factors are key signalling molecules that control and regulate cellular events involved in tissue development, homeostasis and repair eliciting autocrine, paracrine and endocrine effects at very low concentrations (picogram range) (Lazar-Molnar *et al.* 2000, Smith *et al.* 2012). These molecules act by binding to specific cell surface receptors and initiating a cascade of intracellular events, leading to transduction of the signal to the cell nucleus; this, in turn, may result in gene expression changes that ultimately influence cell behaviour and activity. To date, several growth factors have been identified in dECM including transforming growth factor-beta 1 (TGF-β1) (Finkelman *et al.* 1990, Cassidy *et al*. 1997, Smith *et al.* 1998), insulin-like growth factor I and II (IGF-I and II) (Finkelman Mohan *et al.* 1990), bone morphogenetic protein (BMP) (Bessho *et al.* 1991) platelet-derived growth factor – AB (PDGF-AB), vascular endothelial growth factor (VEGF), placental growth factor (PlGF), fibroblast growth factor – 2 (FGF-2) and epidermal growth factor (EGF) (Roberts-Clark & Smith 2000). Pro- and anti-inflammatory cytokines including IL-1α, IL-1β, IL-4, IL-6, IL-8 and IL-12 have also been reported as being present in dECM (Cooper *et al.* 2010). These studies have all used EDTA or Guanidine Hydrochloride to extract dECM components with the likely intention of solubilising them for further analysis rather than for representing a therapeutic process seen clinically. To mimic

therapeutic procedures that induce pulpal repair, calcium hydroxide and MTA have been shown to release TGF-β1, adrenomedullin (ADM) and hepatocyte growth factor (HGF) (Graham *et al*. 2007, Tomson *et al.* 2007, Tomson *et al.* 2013) from dentine matrix *in vitro*. . HGF was shown to induce chemotaxis, differentiation and proliferation in rat dental pulp cells (RDPCs) (Tomson *et al.* 2013). Although a number of growth factors and cytokines present in dECM have been identified, it is likely that there exist many more key signalling molecules sequestered within it that have yet to be reported. Determining the growth factors that are released by therapeutic materials known to induce pulp repair and the mechanism by which they do it will deepen our understanding and improve development of treatment modalities for the injured pulp.

Following tissue injury, a complex series of events, involving various intra- and extra-cellular signalling, are initiated with the aim of promoting tissue repair if favourable environmental factors predominate (Gurtner *et al*. 2008). Wound repair follows a sequence of overlapping stages that include cell homing of progenitor cells to the site of injury, cellular differentiation, proliferation and deposition of extracellular matrix (Kang *et al.* 2012, Smith Smith *et al.* 2012). The necessary cues and signals required for progenitor cell recruitment and proliferation are key events.

Bioactive molecules released from dentine have the potential to initiate the cellular events, which may lead to regeneration of pulp and dentine. Therefore this study aimed to i) characterise growth factors that are sequestered in dentine and are liberated by calcium hydroxide and white and grey MTA, ii) assess the change in composition of dentine following exposure to these materials, and iii) determine if the liberated dECM components promote chemotaxis and growth of dental pulp cells.

Material and Methods

Solution preparation

Solutions of 0.02 M calcium hydroxide (pH 11.9) (Sigma-Aldrich, UK), the solubilised components of white MTA (pH 11.7) (Dentsply Tulsa Dental, USA) and grey MTA (pH 11.7) (Dentsply Tulsa, Tulsa, OK, USA) were prepared by dissolving solids in distilled water. The solutions of MTA were prepared by mixing 1.72 g of white or grey MTA with 1 litre of distilled water with constant agitation at 37 °C for 72 hours. Insoluble particles were subsequently removed by filtration (Tomson *et al.* 2007). Protease inhibitors, 10 mM n-ethylmaleamide (Sigma-Aldrich, Gillingham, UK) and 5 mM phenyl-methyl-sulphonyl fluoride (Sigma-Aldrich) were added to solutions to prevent protein denaturation.

Isolation of dECM components

Dentine was exposed to solutions of calcium hydroxide, white and grey MTA to investigate matrix solubilisation using an established technique (Smith & Leaver 1979, Tomson Grover *et al.* 2007). In brief, powdered dentine ($\leq$0.251 mm^2) was prepared from the crowns and roots of non-carious intact permanent human extracted molars and premolars with ethical approval from the UK National Research Ethics Service (09/H0405/33). The dentine was divided into 5g aliquots and exposed to 20mL of one of the three solutions for 14 days with constant agitation at 4 °C. The pulp capping solution was replaced daily following centrifugation (Jouan B4i, Thermo scientific, Waltham, MA USA) at 3026 x g for 10 minutes. Supernatants were decanted and pooled. Daily absorbance values were measured at 280 nm using a spectrophotometer (UV/VIS spectrophotometer, Philips, Cambridge, UK) to monitor protein dissolution. The pooled supernatants were exhaustively dialysed for 14 days at 4 °C and dialysates were lyophilised using a freeze dryer (Modulyo, Edwards, Crawley, UK).

Multiplex sandwich ELISA analysis

Based on a previously performed cytokine array (unpublished data), a customised quantitative multiplex sandwich ELISA kit (Quantibody® RayBiotech, Norcross, GA, USA) was employed to determine the concentration of specific growth factors in each dentine extract (table 1). Three replicates of each lyophilised dentine extract were dissolved in PBS at a concentration of 190 µg/mL total protein, as determined by the Bradford assay (Bradford 1976). The multiplex sandwich ELISA kit was used according to the manufacturer's instructions. Fluorescence was measured at a wavelength of 532 nm using a microarray scanner (Agilent G2505B, Craven Arms, UK) at a resolution of 2 µm and concentrations of each growth factor or binding protein were calculated from standard curves.

Fourier-transformed infrared spectroscopy (FTIR)

FTIR was used to determine compositional changes of human dentine powder following dECM component extraction with pulp capping solution. Samples of powdered human dentine were either treated with water (control) or exposed to the calcium hydroxide, white and grey MTA solutions as described and were dried at room temperature. Five replicates of each control or treated sample of dried dentine powder were analysed using a Nicolet 6700 FTIR machine (Thermo Scientific Instruments Corp., Madison, WI, USA) and Omnic 8 software suite (Thermo Scientific Instruments Corp.). The data acquired for each biological replicate were combined, and the resulting spectrum represented the mean of each sample within the mid-IR spectrum (range: 1700 to 800 cm^{-1}) at a resolution of 0.482 cm^{-1}.

Culture Medium

Alpha minimum essential medium (αMEM) (Biosera, Uckfield, UK), containing 2 mM L-glutamine (Sigma-Aldrich) supplemented with 1% penicillin / streptomycin (10 000 units/mL of penicillin with

10 mg/mL streptomycin) (Sigma-Aldrich) and 10% foetal bovine serum (FBS) (Biosera), was used for the culture of RDPCs.

Isolation of primary rat dental pulp cells (RDPCs)

RDPCs were isolated using the Trypsin / EDTA enzyme digestion method previously described (Patel *et al.* 2009). In brief, pulp tissue from the incisors of freshly sacrificed male Wistar Hannover rats (Charles River Laboratories) was mechanically minced using a scalpel to produce pieces no greater than 1 mm^3 and then exposed to 0.25% (w/v) Trypsin 1 mM EDTA.4Na (Gibco, Paisley, UK) at 37 °C in 5% CO_2 for 30 minutes whilst agitated constantly. The tissue suspension was passed through a cell strainer (pore size 70 μm) (Becton Dickenson, Oxford, UK) to obtain a single cell suspension. Isolated cells were seeded in a 25 cm^2 flask. Cells numbers were expanded and all subsequent experiments were performed at culture passage two.

Cell growth assay

To determine the effects of dECM components on pulp cell proliferation, RDPCs were seeded at a density of 1×10^4 in 30 μL of medium in a 48-well plate (IWAKI, Chiba, Japan). RDPCs were allowed to attach for 2 hours prior to addition of 170 μL of medium without FBS. At 24 hours, the medium was replaced with medium containing dECM preparations extracted using calcium hydroxide white and grey MTA, at concentrations of 0 (control), 0.1, 1.0, 10.0 and 100.0 μg/mL. Cultures were maintained for up to 7 days, and 4 replicates for each control and experimental condition were analysed. At each time point (3, 5, and 7 days), cell growth was determined using the WST-1 assay. In short, 20 μL of WST-1 (Roche Applied Biosciences, Mannheim, Germany) was added to 180 μL of medium in each well. Plates were kept in the dark for exactly 1 hour and incubated in standard culture conditions. The absorbance of the reduced compound was measured at a wavelength of 450 nm, with a reference filter at 630 nm, using an ELx800 Absorbance Microplate Reader (Biotek, Winooski, VT, USA). Cell number was calculated from a standard curve.

Chemotaxis transwell assay

To determine the chemotactic ability of dECM extracts migration assays were performed using a two chamber, 96-well plate assay system with 8 μm pore size and hydrophilic membrane (ChemoTx, Neuro Probe, Gaithersburg, MD, USA). RDPCs at a concentration of 1.0×10^6 per mL were labelled using 5 μg/mL of calcein AM (Biotium Inc, Hayward CA, USA). The lower chamber was prepared either with medium containing 10% FBS (positive control), 0% FBS (negative control) or dECM preparations extracted using calcium hydroxide and white and grey MTA at concentrations between 0.1 - 100 μg/mL. 30 μL of labelled cell suspension was pipetted carefully onto the upper chamber and the plate was incubated for 45 minutes at 37 °C in 5% CO_2. The number of cells that had migrated to the lower chamber was determined using a microplate fluorometer (Twinkle LB970, Berthold Technologies, Harpenden, UK) at an excitation wavelength of 494 nm and emission of 517 nm. Cell number was calculated from a standard curve and four biological replicates were used for each condition.

Statistical analysis

Statistical differences between the experimental and control groups were determined using one way ANOVA with Bonferroni post hoc analysis carried out using SPSS software (SPSS Inc, Chicago, IL, USA) with $p < 0.05$ deemed to be statistically significant.

Results

Growth factors liberated by solutions of calcium hydroxide, white and grey MTA

Several members of different growth factor families were solubilised by calcium hydroxide, white and grey MTA (Fig. 1). Of the ten growth factors analysed, nine were detected in extracts released by white and grey MTA and six in those solubilised by calcium hydroxide. Six cytokines were present in one or more of the extracts that have not been previously reported to present or released from in dentine; these include: SCF, M-CSF, GM-CSF, IGFBP-1, NGF, GDNF.

VEGF was present in all extracts, with white and grey MTA extracts liberated significantly greater concentration ($p \leq 0.0001$) than the calcium hydroxide solution. SCF, not previously identified in dentine, was only extracted by the grey MTA solution. IGF-I was detected in extracts obtained using the white MTA (10119 ±1122.4 pg/mL) and grey MTA (9128.7 ±483.9 pg/mL) solutions, but were not liberated by calcium hydroxide. IGF-II was extracted by all agents; calcium hydroxide (1042.6 ±39.8 pg/mL), white MTA (210.3 ±61.1 pg/mL) and grey MTA (353.07 ±47.7 pg/mL). The concentration of IGFBP-1 released from dentine was similar for all extracting agents. The colony-stimulating factor, M-CSF, was extracted by white and grey MTA in relatively low concentrations (4.1-5.75 pg/mL) but not at all by calcium hydroxide. GM-CSF was extracted by calcium hydroxide and white MTA but not by grey MTA. No significant differences between concentrations extracted for GM-CSF were detected. Analysis of neurotrophic growth factors (GDNF, NGF) revealed that NGF was detected in extracts released by white MTA (1.15 ±0.4 pg/mL) and grey MTA (2.05±0.06 pg/mL), however it was not detected in the calcium hydroxide extract. GDNF was detected in all extracts; the highest concentration was present in the extract released by calcium hydroxide (233.43 ±55.4 pg/mL), significantly greater than white MTA (98.3 ±3.9 pg/mL) ($p=0.02$) and grey MTA (63 ±2.9 pg/mL) ($p<0.0001$). EGF was detected at relatively low levels in all samples of dECM components.

Dentine composition following exposure solutions of calcium hydroxide, white and grey MTA

To provide an insight into compositional changes in dentine following dECM component extraction by calcium hydroxide, white and grey MTA solutions FTIR was used. The FTIR plots are shown in Fig. 2. Treatment with these solutions shows little effect on phosphate bands between 1180 - 885 cm^{-1} but does, however, demonstrate disruption of the organic components with flattening of amide bands between 1700-1200 cm^{-1}.

Effects of pulp capping solution dECM extracts pulp cell growth and chemotaxis

To determine the effect of solubilised dentine components on primary RDPCs, in terms of their potential capacity to stimulate cell growth in the proliferative phase of wound healing processes, the WST-1 (Roche Applied Biosciences) assay was applied (Fig. 3). The general trend was that medium supplemented with dentine extracts increased cell numbers of RDPCs at concentrations between 0.1 μg/mL and 10 μg/mL over a 7-day period. There was no increased cell numbers at the higher concentration of 100 μg/mL. On day 5, RDPCs exposed to dECM components extracted by white MTA had significant differences at concentrations of 1.0 μg/mL (43,000 cells, p=0.013), 10 μg/mL (43,500 cells, p=0.01) and 100 μg/mL (42,000 cells, p=0.027) when compared with the control. On day 7, RDPC numbers increased in a dose-dependent manner when exposed to increasing concentrations of white MTA-extracted dECM components up to 10 μg/mL: 0.1 μg/mL (48,500 cells), 1.0 μg/mL (53,000 cells), 10 μg/mL (58,500 cells) compared with control (43,000 cells). Comparison with controls demonstrated that the 10 μg/mL dECM exposure was significant (p=0.014). RDPC exposed to dentine components extracted by grey MTA showed a similar pattern on day 7, however, maximum cell numbers were detected at a lower concentration of 1.0 μg/mL which was significant when compared with control (p=0.05).

The chemotactic potential of dECM components was assessed using a transwell plate assay (Fig. 4). Medium supplemented with FCS was used as a positive control and demonstrated significantly more chemoattraction of RDPCs compared with medium without FCS. Medium supplemented with dECM

components at concentrations of 0.1 μg/mL to 100 μg/mL extracted with either calcium hydroxide, white or grey MTA had significantly higher chemotaxis levels for RDPCs compared with medium with no supplement. Dentine components extracted with calcium hydroxide revealed that both 1.0 μg/mL and 10 μg/mL induced significantly more RDPC migration than the lower concentration of 0.1 μg/mL (p=0.022 and p=0.004, respectively). For dentine components extracted with white MTA, the highest concentration tested (100 μg/mL) induced significantly more cell migration than the 0.1 μg/mL (p=0.04) supplement.

Discussion

It is well established that healing of the wounded pulp can be induced through material-directed repair (Goldberg *et al.* 2008) and it has been suggested that the interaction of the materials with dentine may release growth factors involved in repair of the wounded pulp (Graham *et al.* 2006, Tomson *et al.* 2007). When used clinically, it is inevitable that calcium hydroxide, white and grey MTA will interact with local tissue fluids subsequently altering the local aqueous environment at the tissue / material interface. The *in vitro* model used here is a well-established technique that has been adapted to mimic these interactions in the context of examining effects on dentine matrix dissolution. Quantitative multiplex ELISA analysis of dentine components released by calcium hydroxide, white and grey MTA demonstrates release of a rich cocktail of growth factors, a number of which that have not been previously reported. These include: SCF, M-CSF, GM-CSF, IGFBP-1, NGF, GDNF. The relatively broad analysis of growth factors solubilised by the pulp-capping materials used here demonstrates that each material releases a different profile of bioactive molecules. Indeed white and grey MTA liberate a broader profile of nine bioactive molecules compared with the six released by calcium hydroxide. Furthermore differences exist between the bioactive molecular profile of white and grey MTA. The electrolytic compositions of the ionic dissolution products from these materials create environments within which bioactive molecules bound to matrix or mineral are consequently released in a differential manner. Further studies should now seek to characterise differences in the ionic dissolution product profiles between these materials to better understand their mechanistic basis of actions. The broader profile of bioactive molecules released by white and grey MTA may suggest why MTA induces a more favourable pulp response when used as a pulp capping agent than calcium hydroxide (Nair *et al.* 2008)

The bioactive molecules released from dentine by calcium hydroxide, white and grey MTA have all previously been shown to play an important role in tissue development, repair or homeostasis. Those growth factors that have not previously been reported, such as the angiogenic growth factor SCF, which was detected in grey MTA extracts, are particularly interesting. SCF has been shown to exert

its biological functions by binding to, and activating, the receptor tyrosine kinase c-Kit and has been demonstrated to play an important role in haematopoiesis, amongst other physiological functions (Lennartsson & Ronnstrand 2012). SCF can induce liberation of primitive haematopoietic cells from the bone marrow into the blood (Fleming *et al.* 1993, Yan *et al.* 1994) and is used therapeutically to enhance the release of haematopoietic stem cells as an adjunctive therapy in patients undergoing chemotherapy and/or radiotherapy. Chemotaxis of cells involved in tissue repair is a fundamental process in the healing of any tissue; with evidence emerging that that cells involved in pulp tissue regeneration may not, in fact, only originate from local tissue niches, but may be haematopoietic or perivascular in origin (Feng *et al.* 2011, Frozoni *et al.* 2012). Indeed SCF may play an important role in pulp repair as it has recently been shown that SCF is a powerful promotor of the induction of migration, proliferation, and chemotaxis of DPCs (Pan *et al.* 2013).

M-CSF and GM-CSF were detected in dentine extracts solubilised by pulp-capping agents. Calcium hydroxide, however, did not solubilise M-CSF, and GM-CSF was not solubilised by grey MTA. The CSF group of glycoproteins stimulate the proliferation, differentiation, and survival of haematopoietic cells as well as activating mature myeloid cell functions (Hamilton 2008). It has recently been shown that M-CSF induces resident tissue macrophages to differentiate and proliferate in the dental pulp, rather than a source of such cells being derived from circulating precursors (Iwasaki *et al.* 2011) as was previously thought. Secretion of GM-CSF and osteopontin at the pulp-dentine interface, by immunocompetent cells such as macrophages and dendritic cells, locally induces maturation of dendritic cells, thus encouraging increased activity of odontoblasts and their differentiation from pulpal progenitors (Saito *et al.* 2011). Inflammatory cytokines have been shown to be present in dentine components released by lactic acid and calcium hydroxide (Cooper *et al.* 2010) and emerging evidence indicates that low level immune / inflammatory processes, induced by dental injury, may contribute to the regenerative mechanisms leading to pulpal repair (Cooper *et al.* 2014).

Both IGF-I and IGF-II have previously been shown to be present within the dECM (Finkelman *et al.* 1990), consistent with the work presented here. IGFs operate within a system often referred to as the

IGF-axis, which includes six binding proteins (IGFBP 1-6). The IGF-axis reportedly plays an important role in cell growth, differentiation and apoptosis in many different tissues (Jones & Clemmons 1995). The discovery of IGFBP-1 in dentine extracts and its release by pulp-capping agents may suggest that IGFBPs may be involved in modulation of bioactivity induced by IGF-I and II, either by attenuating their action or by prolonging their half-life (Arai *et al.* 1996; Kuang *et al.* 2006).

NGF and GDNF were solubilised from dentine matrix by both white and grey MTA. NGF has previously been shown to induce mineralisation and increase expression of Dentine sialophosphoprotein (DSPP) and Dentine matrix protein-1 (DMP-1) *in vitro* in cells with an odontoblast lineage (Arany *et al.* 2009). Interestingly, there appears to be interplay between NGF and TGF-β1 in pulp repair. TGF-β1 is known to play an important role in pulp repair and is solubilised from dentine matrix by white and grey MTA (Tomson *et al.* 2007). TGF-β1 increases expression and secretion of NGF in a dose-dependent manner in human pulp cells (Srisawasdi & Pavasant 2007). These latter data suggest that, in the event of pulpal injury, the abundance of TGF-β1 may induce increased secretion of NGF (Yongchaitrakul & Pavasant 2007). Another neurotrophic factor shown to be in dentine matrix, and released by calcium hydroxide and white MTA, was GDNF. Recently, it has been demonstrated *in vitro* that GDNF may have multi-functionality within the dentine-pulp complex, acting as both a cell survival factor and mitogen during tooth injury and repair (Gale *et al*. 2011).

The *in vitro* experiments presented here aim to mimic the interaction of dentine with local tissue fluids that will have a modified electrolytic composition when pulp capping agents are used clinically. The materials investigated may create an environment in which ion exchange occurs between the dentine and the tissue fluid, such that, bioactive matrix bound non-collagenous proteins are consequently released. This is possible because of the existence of a metastable hydrated layer of loosely-bound ions on the crystalline surface of mineralised matrix that readily allows ion exchange (Cazalbou *et al.* 2005). Although the profile of growth factors released by each pulp capping agent tested was different, FTIR spectra shows that dentine treated with calcium hydroxide, white and grey

MTA results in little change in composition. There is partial removal of amide bands I, II and III (1700 – 1300 cm^{-1}) but no change in PO_4^{3-} group peak (1000–1100 cm^{-1}) suggesting growth factor liberation without removal of the major inorganic component of dentine. It is proposed that this mechanism may allow a gradual release of bioactive dentine components as seen during these *in vitro* extractions by pulp-capping agents. MTA materials undertake this mechanism more efficiently and release a broader profile of bioactive molecules than calcium hydroxide and this may be a contributing factor to why MTA materials are more successful at inducing pulp tissue repair than calcium hydroxide (Aeinehchi *et al.* 2003, Chacko & Kurikose 2006, Nair *et al.* 2008).

The rich cocktail of growth factors shown to exist in dentine matrix and shown to be released by the pulp-capping agents here potentially directly contribute to modulating the cascade of cellular events required in repair of the pulp. Dentine components solubilised by calcium hydroxide, white and grey MTA were all shown to be powerful chemotactic agents for RDPCs. When RDPCs were exposed to different concentrations of dentine components, cell growth was stimulated in a dose-dependent manner at low to moderate concentrations. These data are also consistent with previous reports (Musson *et al.* 2010, Zhang *et al.* 2011). It is anticipated that pulp cells derived from other species, including human, would behave in a similar manner to the rat cells studied here. The solubilised dentine components investigated here contain a heterogeneous mixture of biologically active constituents, only some of which have been characterised in the present study, and a variety of bioactive molecules have previously been reported (Graham *et al.* 2007, Tomson *et al.* 2007, Smith et al. 2012, Tomson *et al.* 2013). Because of the broad range of bioactive molecules released from dentine, the cellular responses studied here cannot be attributed to any single molecule; further functional studies involving molecular deletions will help to clarify this. It has been demonstrated that dECM components, liberated by the soluble products of therapeutic pulp-capping agents, induce migration and proliferation in dental pulp cells which may suggest that the solubilisation of bioactive molecules from dentine by these agents may promote events involved in reparative dentinogenesis.

Conclusion

These analyses have identified a number of new growth factor families in dentine and have demonstrated that it is a richer reservoir of potent signalling molecules than previously thought. It has also shown that white and grey MTA release a broader profile of signalling molecules than calcium hydroxide and that all dentine components released by these agents contribute to functional events in wound healing.

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Table 1: A list of the proteins (with abbreviations) analysed in dECM components extracted by calcium hydroxide, white MTA and grey MTA using a customised multiplex sandwich ELISA.

Vascular endothelial growth factor (VEGF)	Stem cell factor (SCF)
Insulin-like growth factor – I and II (IGF-I and IGF-II)	Insulin-like growth factor binding protein 1 (IGFBP-1)
Macrophage colony stimulating factor (M-CSF)	Granulocyte macrophage colony stimulating factor (GM-CSF)
Nerve growth factor (NGF)	Glial cell-derived neurotrophic factor (GDNF)
Epidermal growth factor (EGF)	

Figure Legends

Figure 1: Mean concentration of growth factors in dECM components released from human dentine by solutions of calcium hydroxide, white MTA and grey MTA as determined by multiplex ELISA (±SD, n=3). * indicates statistically significant difference (p<0.05) using one way ANOVA.

Figure 2: FTIR spectra (1700 - 800 cm−1) of dried powdered human dentine samples (n=5) that were A) untreated (control) or exposed to solutions of B) calcium hydroxide, C) white MTA or D) grey MTA over a 14-day period during dECM components extraction.

Figure 3: RDPC growth over 7 days following exposure to a range of concentrations of dECM components extracted by A) calcium hydroxide, B) white MTA or C) grey MTA as determined by the WST-1 assay. Error bars represent one standard deviation from the mean for quadruplicate analyses. *p≤0.05 when compared with control using one-way ANOVA.

Figure 4: RDPC migration induced by A) calcium hydroxide, B) white MTA and C) grey MTA extracted dECM components at concentrations between 0.1 - 100 μg/mL. Positive control (FBS +ve) included 10% FBS and negative control was medium without FBS (FBS -ve). All experiments were performed in quadruplicate and data are expressed as mean values ±SD. *p≤0.05, **p≤0.001 when compared with negative control using one-way ANOVA.
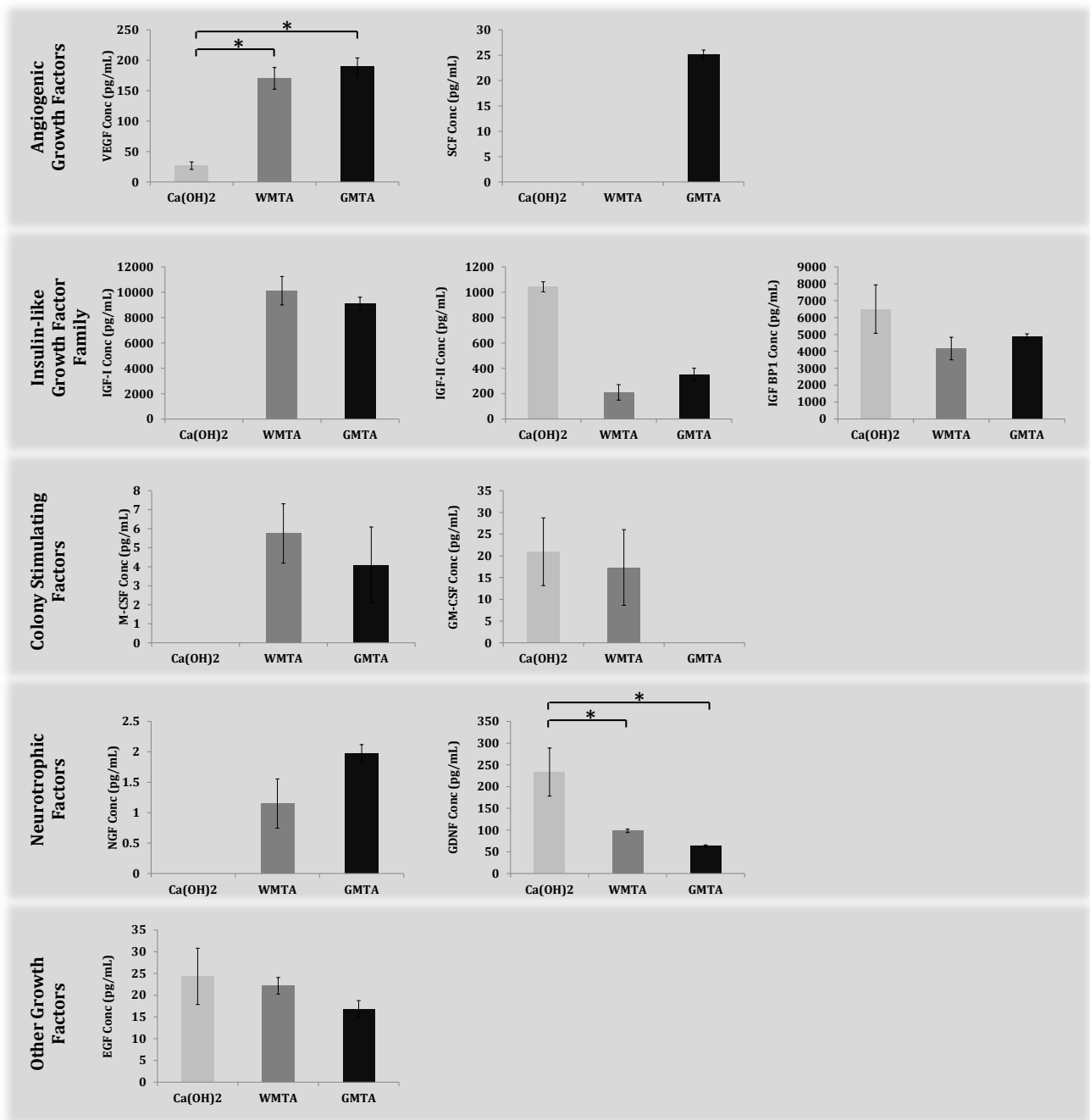
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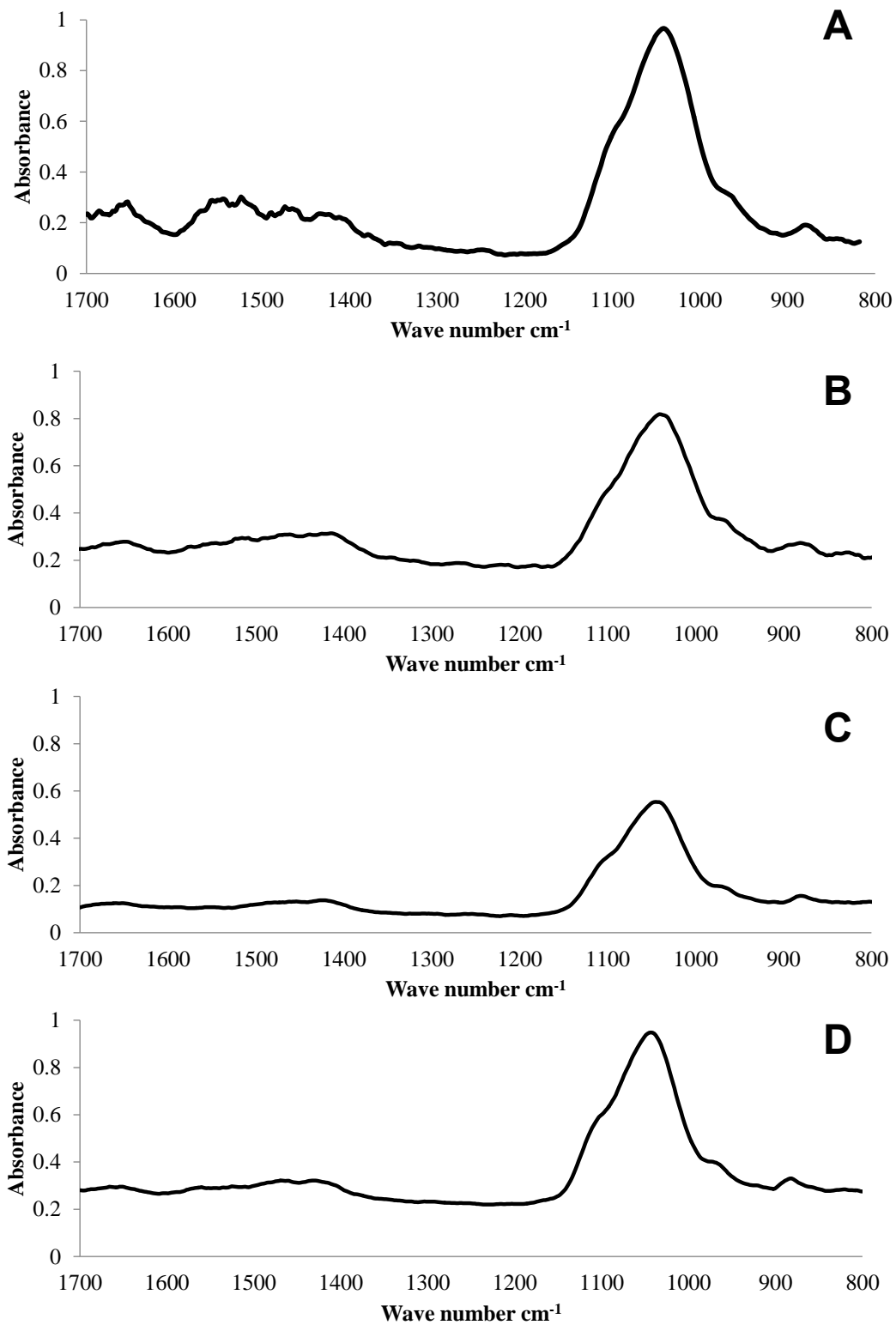
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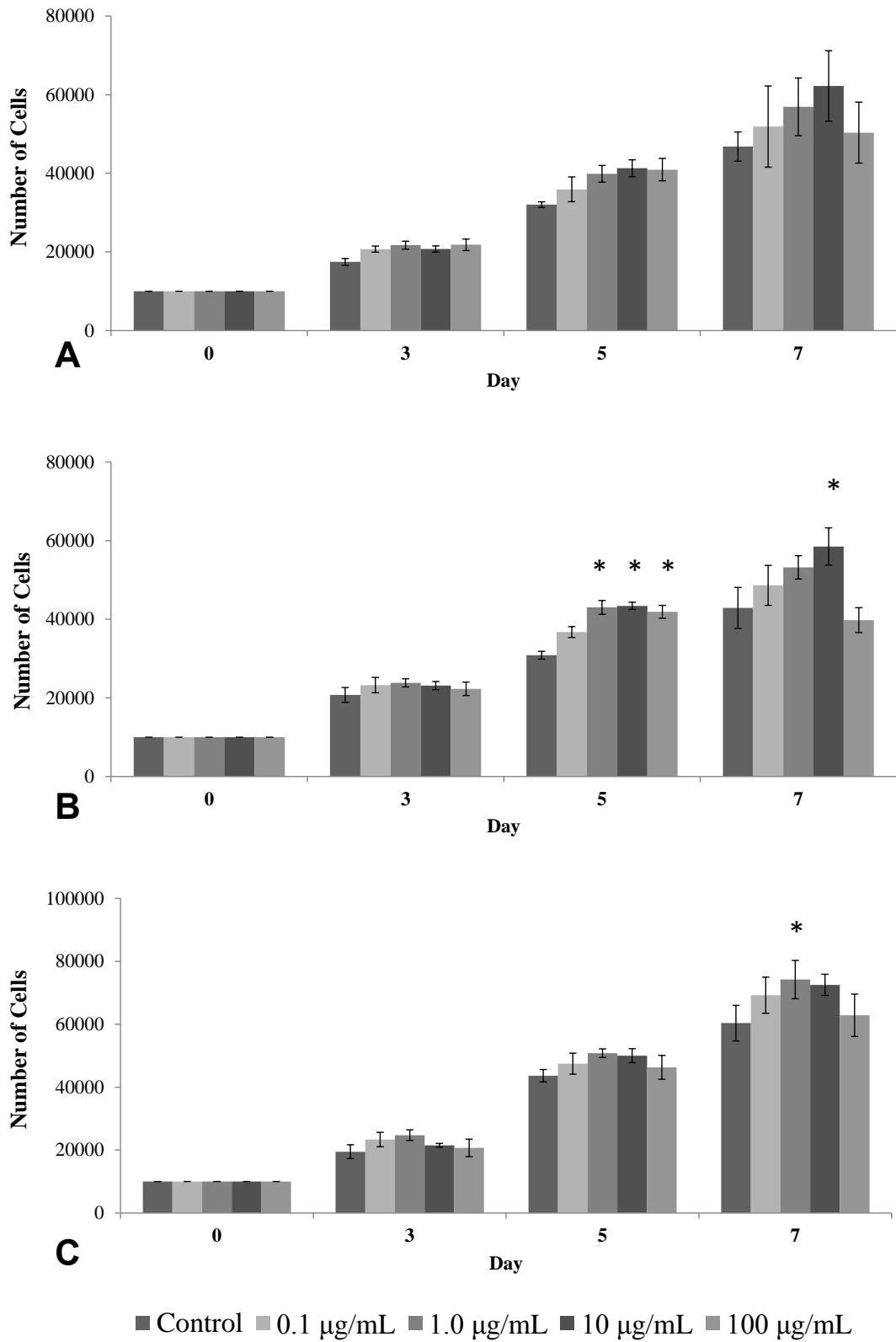
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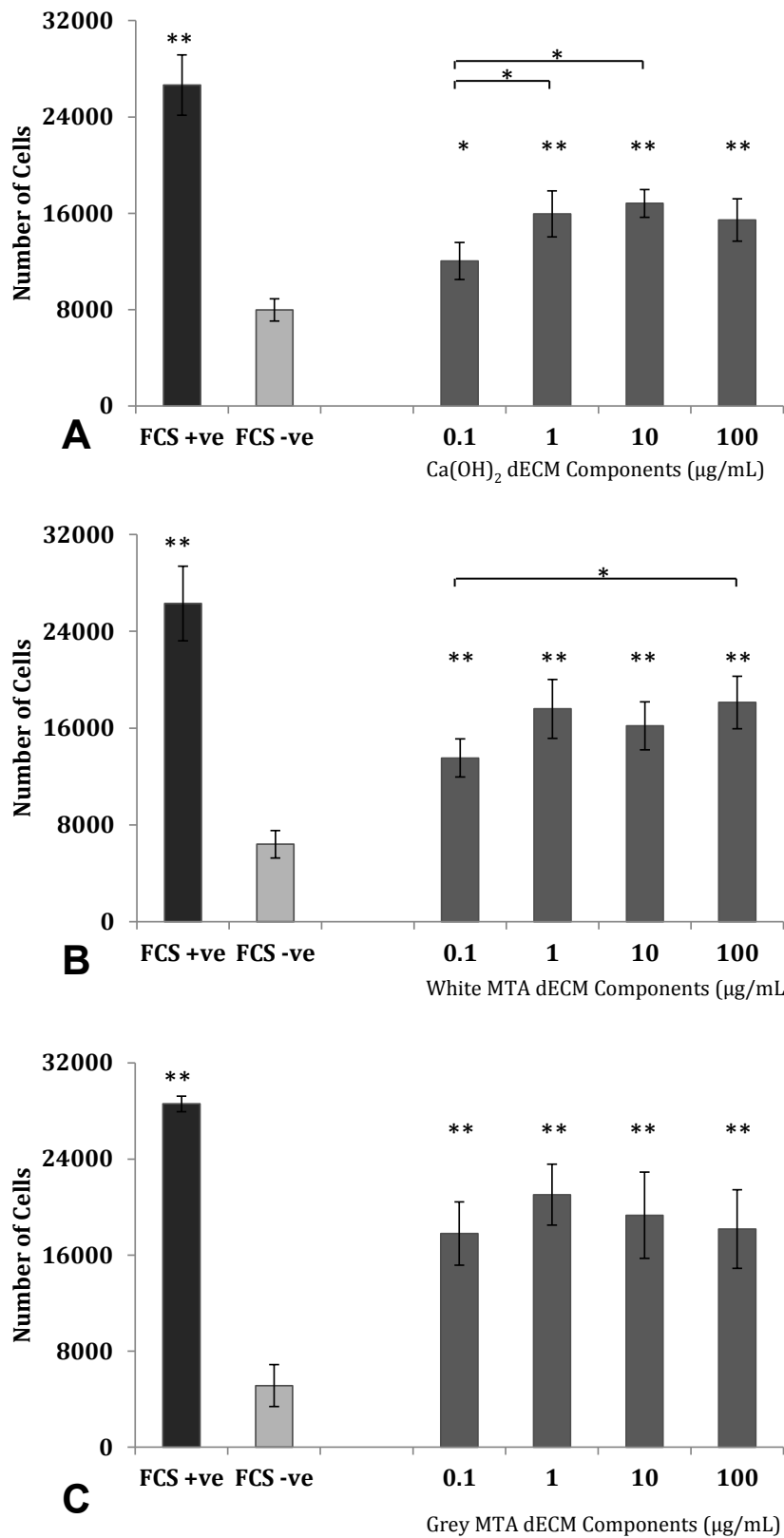
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