International Endodontic Journal

doi:10.1111/j.1365-2591.2011.01890.x

Human dental pulp cell responses to new calcium silicate-based endodontic materials

C. C. Chen[1,2], M. Y. Shie[3] & S. J. Ding[1,4]

[1]Department of Dentistry, Chung Shan Medical University Hospital, Taichung City; [2]School of Dentistry, Chung Shan Medical University, Taichung City; [3]Institute of Biomedical Engineering, National Cheng-Kung University, Tainan City; and [4]Institute of Oral Biology and Biomaterials Science, Chung Shan Medical University, Taichung City, Taiwan

Abstract

Chen CC, Shie MY, Ding SJ. Human dental pulp cell responses to new calcium silicate-based endodontic materials. *International Endodontic Journal*, **44**, 836–842, 2011.

Aim To evaluate human dental pulp cell responses to radiopaque dicalcium silicate cement and white-coloured mineral trioxide aggregate (WMTA).

Methodology Flow cytometry was employed to quantify the phase percentage of pulp cell cycle. Alamar Blue was used for real-time and repeated monitoring of cell proliferation. Reverse transcription-polymerase chain reaction was performed to determine gene expression in pulp cells cultured on the cements.

Results The cells cultured on the radiopaque dicalcium silicate cement had similar S and G2 phases in the cell cycle and proliferation to WMTA at all culture times. In addition, the two materials presented the same evolution with similar values in interleukin-1, inducible nitric oxide synthase, alkaline phosphatase, osteocalcin and bone sialoprotein gene expression at all culture times.

Conclusions The dental pulp cell responses to radiopaque dicalcium silicate cement were similar to those reported for WMTA in terms of cell cycle, proliferation, immunocompatibility and osteogenic differentiation.

Keywords: calcium silicate cement, dental pulp cell, gene expression, mineral trioxide aggregate, root-end filling material.

Received 12 January 2011; accepted 6 April 2011

Introduction

A variety of calcium silicate-based materials such as Portland cement (Ribeiro *et al.* 2005, Islam *et al.* 2006, Shahi *et al.* 2010, Zhang *et al.* 2010) and mineral trioxide aggregate (MTA) (Camilleri & Pitt Ford 2006, Islam *et al.* 2006, Gomes-Filho *et al.* 2009, Ding *et al.* 2010a, Shahi *et al.* 2010) have been developed for use in dentistry. MTA has positive characteristics such as favourable tissue reactions and the induction of mineralized repair tissue (Gomes-Filho *et al.* 2009). Hence, it has been recommended for pulp capping (Aeinehchi *et al.* 2003), pulpotomy (Moretti *et al.* 2007), apical barrier formation in teeth with open apexes (Felippe *et al.* 2006), repair of root perforations (Bargholz 2005), root canal filling (Torabinejad & White 1995) and root-end filling (Chiang & Ding 2010).

In previous studies (Chen *et al.* 2009a,b), a quick-setting, aluminium-free, dicalcium silicate cement was successfully prepared using a sol–gel method. The cement could quickly form 'bone-like' apatite spherulites after immersion in a simulated body fluid (Chen *et al.* 2009b). After the addition of Bi_2O_3, the radiopaque dicalcium silicate cement sets within 24 min when mixed with water, which was significantly lower than that of white-coloured mineral trioxide aggregate (WMTA) (168 min) (Chiang & Ding 2010). Additionally, MG63 cell viability cultured on radiopaque dicalcium silicate cement was higher than that cultured on WMTA at all culture times (Chiang & Ding 2010), suggesting that the cement could be applied in endodontic treatments. The further investigation of osteogenecity is necessary because the responses of

Correspondence: Shinn-Jyh Ding, Institute of Oral Biology and Biomaterials Science, Chung Shan Medical University, Taichung City, Taiwan (Tel.: +886-4-24718668 ext.55529; fax: +886-4-24759065; e-mail: sjding@csmu.edu.tw).

various cell types to a material are different in various cell culture environments. Human dental pulp cells have the ability to differentiate along the osteoblast lineage and contribute to the dentinal regeneration process (Gronthos *et al.* 2000). According to the literature (Moghaddame-Jafari *et al.* 2005), MTA induces a slight increase in the proliferation of odontoblast-like cells and undifferentiated pulp cells. Further, they are more suitable than MG63 cells for evaluating the bone tissue response to cement materials.

Following previous studies (Chen *et al.* 2009a,b, Chiang & Ding 2010), the purpose of this study was to examine the responses, including cell cycle, immune response and osteogenic differentiation of human dental pulp cells to radiopaque dicalcium silicate cement in comparison with those to WMTA.

Materials and methods

Specimen preparation

Reagent-grade tetraethyl orthosilicate ($Si(OC_2H_5)_4$; Sigma-Aldrich, St. Louis, MO, USA) and calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$, Showa, Tokyo, Japan) were used as precursors for SiO_2 and CaO, respectively. The catalyst was 2 mol L^{-1} nitric acid, and absolute ethanol was used as the solvent. The molar ratio of $Ca(NO_3)_2{\cdot}4H_2O$ to $Si(OC_2H_5)_4$ was 3:2. General sol–gel procedures, such as hydrolysis and ageing, were adopted. A detailed description of the powder's fabrication has been reported (Chiang & Ding 2010). After sintering at 800 °C for 2 h with a high-temperature furnace, the sintered granules were then ball-milled for 12 h in ethyl alcohol using a centrifugal ball mill (Retsch S 100, Hann, Germany) and then dried in an oven at 60 °C. Bi_2O_3 (Sigma-Aldrich) was added to the ground powder at 20 wt%, as described by Torabinejad & White (1995), using a conditioning mixer (ARE-250, Thinky, Tokyo, Japan). The cement specimens with 20 wt% Bi_2O_3 were hand-mixed at a liquid-to-powder (L/P) ratio of 0.4 mL g^{-1}. White-coloured ProRoot MTA (Dentsply Tulsa Dental, Tulsa, OK, USA) was used as an experimental group. The liquid phase was water, and an L/P ratio of 0.3 mL g^{-1} was used according to the manufacturer's instructions. After mixing, the cement fully covered each well of the 24-well plate to a thickness of 2 mm; the specimens were stored in an incubator at 100% relative humidity and 37 °C for 1 day to set.

Cell culture

Human dental pulp cells were freshly derived from a caries-free, intact premolar that was extracted for orthodontic treatment purposes as described previously (Chen *et al.* 2010). The patient gave informed consent. Approval from the Ethics Committee of the Chung Shan Medicine University Hospital was obtained. The tooth was split sagittally with a chisel, and periodontal ligament tissue was then immersed in phosphate-buffered saline (PBS) solution. Pulp tissue was cut into fragments and immersed in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Langley, OK, USA) containing 0.1% collagenase (Sigma) and 0.1% dispase (Sigma) for 1 h. The pulp cells were collected from medium at 360 g for 5 min. The cell pellet was resuspended in DMEM containing 20% foetal bovine serum (Gibco), 100 units mL^{-1} penicillin G and 100 mg mL^{-1} streptomycin in 5% CO_2 at 37 °C. Cells were subcultured by successive passaging at a 1 : 3 ratio. Cell cultures between the fourth and eighth passages were used. Prior to cell incubation, the hardened cement discs were sterilized by soaking in a 75% ethanol solution and exposure to ultraviolet (UV) light for 2 h. Pulp cell suspensions (2×10^4 cells per well) were directly seeded over each specimen, which was then placed in a 24-well plate. Cells cultured on the tissue culture plate without the cement were used as a control.

Cell cycle

The propidium iodide (PI) flow cytometric assay is widely used for the analysis of the cell cycle. The DNA content was quantified to determine the percentage of cells in each cycle phase of subG1, G1, S and G2. After culture for various time periods, floating and adhered cells were collected, centrifuged and fixed with cold ethyl alcohol at −20 °C over night. Cell samples were resuspended in 50 μg mL^{-1} PI (Invitrogen, Grand Island, NY, USA), 0.1% Triton X-100 and 100 μg mL^{-1} RNAse A (Sigma-Aldrich) in the dark at 4 °C for 2 h. The resulting nuclei suspension was analysed with a Calibur (Becton Dickinson, Franklin Lakes, NJ, USA) fluorescence activated cell sorter at a 488-nm excitation and with a 560-nm band pass filter for red fluorescence of PI. The phase percentage of pulp cells in the cell cycle was determined using WinMDI 2.8 software (Scripps Research Institute, La Jolla, CA, USA). The average from three different assays was recorded.

Cell proliferation

The reagent alamar Blue (Invitrogen) was used for real-time and repeated monitoring of cell proliferation, which is based on the detection of mitochondrial activity. Briefly, at the end of the culture period, the medium was discarded and washed with PBS twice. Each well was filled with 0.3 mL at a ratio of 1 : 9 alamar Blue/fresh medium and incubated at 37 °C for 3 h. One hundred microlitres of the solution in each well was transferred to a 96-well tissue culture plate. Plates were read in a Sunrise Microtiter Reader (Tecan Austria Gesellschaft, Salzburg, Austria) at 570 nm with a reference wavelength of 600 nm. The results were obtained in triplicate from three separate experiments. Cell proliferation was represented in terms of optical density.

Gene expression analysis

Reverse transcription–polymerase chain reaction (RT-PCR) was used to determine gene expression in human dental pulp cells cultured on the various specimens. The matrices were washed with PBS at days 1, 3, 7 and 15, and the total RNA from the cells was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. The total RNA concentration was quantified by measuring the OD at 260 nm, as well as the OD260/OD280 ratio, using a Beckman DU640B spectrophotometer (Fullerton, CA, USA). PCR primers were designed for various bone formation genes: osteocalcin (OC), collagen type I (COL I), alkaline phosphatase (ALP), bone sialoprotein (BSP) and actin (AC). The primers for interleukin (IL)-1 and inducible nitric oxide synthase (iNOS) were referred to the previous study (Ding *et al.* 2010b). These primers were designed on the basis of published gene sequences (NCBI and PubMed). The mRNA was converted to cDNA using a thermal cycler (GeneAmp® PCR System 9700; Applied Biosystems, Foster City, CA, USA). Each PCR product was analysed by separation with 2% agarose (in Tris-acetate-EDTA buffer) gel electrophoresis and visualized following ethidium bromide staining. The stained bands were photographed using a Syngene bio-imaging system (Frederick, MD, USA). For semi-quantitative analysis of the genes, the photographed bands were analysed with Scion Image software (Scion Corp., Frederick, MD, USA), and the intensity of each band was normalized to that of AC. Semi-quantitative analysis was carried out in three separate sets of experiments.

Statistical analysis

One-way ANOVA statistical analysis was used to evaluate the significance of the differences between the mean values. Scheffé multiple comparison testing was used to determine the significance of the deviations in the data for each specimen. In all cases, the results were considered statistically significant at a P value <0.05.

Results

Cell cycle

In Fig. 1, the phase percentage of cells in the subG1, G1, S and G2 is given as a function of culture time. With increasing culture time, the percentage of cells in the G1 phase appreciably decreased for all test groups, along with S and G2 phases increased. The cells cultured on radiopaque dicalcium silicate cement were not significantly different ($P > 0.05$) from those on WMTA in S and G2 phases at all culture times, but being significantly ($P < 0.05$) higher than the control. Of note, the cell population in the subG1 phase was in the range of 3.1–3.6% for all test groups. No significant difference ($P > 0.05$) in the subG1 phase between radiopaque dicalcium silicate cement and WMTA was found.

Cell proliferation

Alamar Blue showed that the number of cells cultured on either radiopaque dicalcium silicate cement or WMTA surfaces had a significant ($P < 0.05$) higher

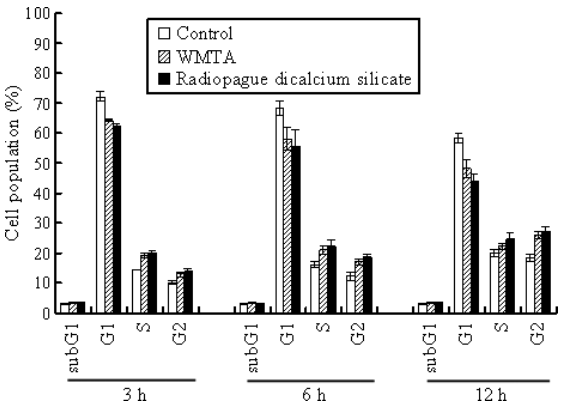

Figure 1 Phase percentage of pulp cell cycle for the specimens at various time-points.

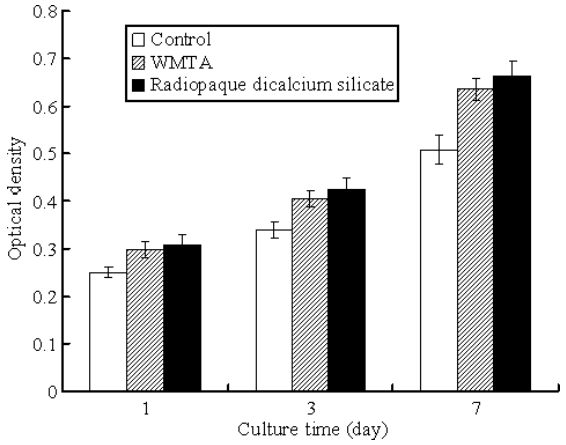

Figure 2 Alamar Blue assay for pulp cell proliferation cultured on the specimens at various time-points.

value amount at all culture times when compared to the control (Fig. 2). However, there were no significant differences ($P > 0.05$) between the two cements at all culture times.

Immune response

No significant differences ($P > 0.05$) between radiopaque dicalcium silicate cement and WMTA were found in either IL-1 (Fig. 3a) or iNOS (Fig. 3b) production, but the immune response of cells on radiopaque dicalcium silicate cement was lower than those on WMTA at all culture times. However, the cells on the surfaces of the two cements had a significantly ($P < 0.05$) higher immune response than those on the control at all culture times, with the exception of 7-day iNOS expression.

Osteogenic differentiation

Changes in the expression of the pulp cell phenotypic markers AC, COL I, ALP, BSP and OC with varying culture durations are shown in Fig. 4. AC was used as an internal control and was produced at a comparable level in control cells and cells grown on the test cements. COL I expression was similar in the control and experimental groups during the culture time periods (Fig. 4a). ALP expression was higher in cells on the two cement surfaces at day 7 than in cells at day 3, but it was slightly downregulated after 15 days in culture (Fig. 4b). No significant differences ($P > 0.05$) in ALP mRNA expression were detected between the two cements for all time-points. BSP (Fig. 4c) and OC (Fig. 4d) secretions increased in pulp cells cultured on either radiopaque dicalcium silicate cement or WMTA with increasing culture time. Similar to ALP, the two gene expressions in cells were not found to be significantly different ($P > 0.05$) between the two cements. Interestingly, the cells cultured on the two cements produced a significantly ($P < 0.05$) higher ALP, BSP and OC levels than those secreted on the control at all culture times.

Discussion

An ideal biomaterial for bone repair and replacement would administer the appropriate signals to direct the processes of osteogenesis, such as cell proliferation, differentiation, matrix deposition and, ultimately, mineralization of the extracellular matrix, along with the lack of apoptosis. Additionally, biomaterials are foreign to the host, and it is thus not surprising that the immune response is an important determinant of

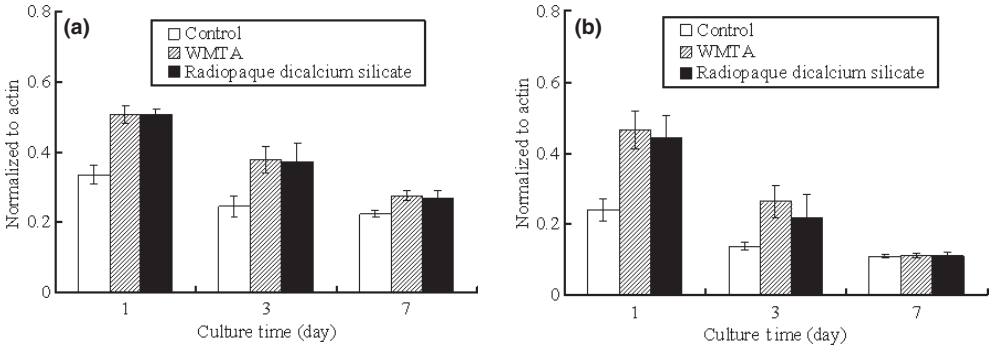

Figure 3 (a) Interleukin (IL)-1 and (b) inducible nitric oxide synthase (iNOS) responses of pulp cells cultured on the specimens at various time-points. The mRNA levels of IL-1 and iNOS were determined by Reverse transcription–polymerase chain reaction and normalized to the corresponding actin mRNA levels.

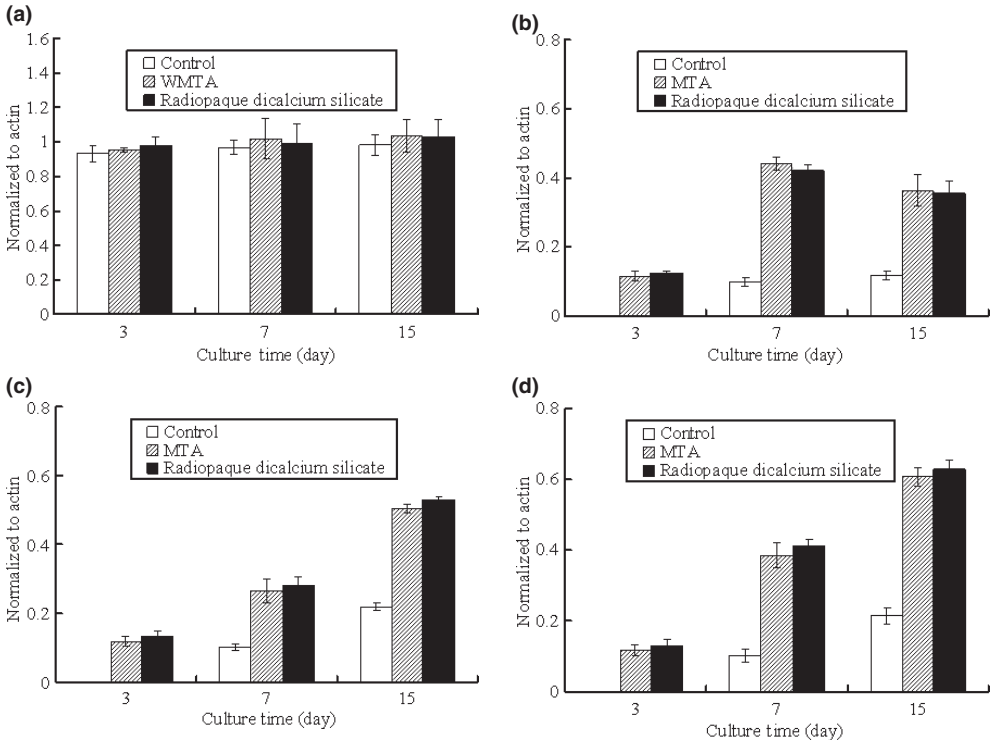

Figure 4 Osteogenic expression levels of pulp cells cultured on the specimens at various time-points. The intensity of each amplified cDNA band was semi-quantified and normalized to that of actin. COL I = collagen I, ALP = alkaline phosphatase, BSP = bone sialoprotein and OC = osteocalcin. (a) COL I, (b) ALP, (c) BSP and (d) OC.

biocompatibility (Vosoughhosseini *et al.* 2008). The nonspecific (innate) immune system is responsible for initiating rapid and general responses against invasion by foreign objects. It is essential to elucidate the effects of the newly developed radiopaque dicalcium silicate cement with shortened setting time on the cell cycle, the immune response and osteogenesis of pulp cells.

The cell cycle is the series of events that takes place in a eukaryotic cell leading to its division and replication (Koulaouzidou *et al.* 2009). Not surprisingly, deregulation of cell cycle control proteins may lead to tumour formation. The subG1 phase represents cell death (Gong *et al.* 1994). The G1 corresponds to the interval between mitosis and initiation of DNA synthesis. DNA replication takes places during the S phase following the G1 phase. The completion of DNA replication is followed by the G2 phase, during which cell growth continues and proteins are synthesized in preparation for mitosis (Cooper 2000). In the current study, there were no significant differences in the percentages of cells in the four phases over time between the two test cement specimens, consistent with results of cell proliferation. Hernandez *et al.* (2005) also found that WMTA did not induce an increase in the percentage of apoptotic cell when compared to the control. WMTA induces the proliferation, and not apoptosis, of pulp cells *in vitro* (Moghaddame-Jafari *et al.* 2005, Paranjpe *et al.* 2010).

Immunocompatibility was evaluated by measuring the expression of IL-1 and iNOS. IL-1 is the first 'immune' cytokine positively identified to be involved in the control of bone turnover. It also provides a typical example of multifunctional cytokines involved in the regulation of the immune response, hematopoiesis and inflammation (Akira *et al.* 1990). Minamikawa *et al.* (2009) found that MTA increased iNOS mRNA expression via activation of the nuclear factor kappa B signalling system. iNOS is only expressed in response to inflammatory stimuli. IL-1 causes activation of the iNOS pathway in bone cells (van't Hof & Ralston 2001). It was found that the lowest levels of the pro-inflammatory cytokine IL-1 and iNOS were expressed by pulp cells cultured on the control at all time-points. The higher IL-1 and iNOS levels in cells on the two

cements may partially be attributed to the initial high pH values (Shahi *et al.* 2010). This is consistent with the results of previous studies (Rezende *et al.* 2008, Vosoughhosseini *et al.* 2008, Shahi *et al.* 2010). Nevertheless, both IL-1 and iNOS expressions decreased with increasing incubation time, approaching the expression of the control at day 7. Gomes-Filho *et al.* (2009) showed that Angelus MTA caused moderate reactions at day 7, which decreased with time. An animal study indicated that Angelus MTA upregulated the adaptive humoural immune responses but had little or no effect on pro- or anti-inflammatory cytokine production (Rezende *et al.* 2008).

Osteoblast differentiation is generally accompanied by ALP expression as well as OC, BSP and COL I productions and *in vitro* mineralization. There were no significant differences in COL I expression between the two cements. ALP gene expression is an early marker of osteoblast differentiation. The secretion of ALP from pulp cells began earlier during culture on the two cement surfaces than on the control surface. It is generally accepted that an increase in the specific activity of ALP in bone cells reflects a shift to a more differentiated state (Aubin *et al.* 1995). Moreover, ALP appears to play a crucial role in the initiation of matrix mineralization, and ALP gene expression is downregulated after the start of mineralization, as shown in both two cements on day 15. OC is a later marker of osteoblast differentiation and is the most abundant noncollagenous bone-matrix protein characteristic of osteoblast synthetic function (Ding *et al.* 2009). BSP is specific to mineralized tissues and is expressed at the onset of bone formation during embryogenesis (Ding *et al.* 2010b). Pulp cells on the radiopaque dicalcium silicate cement and WMTA showed significantly increased BSP and OC expression levels with increases in the culture time. In this study, no noticeable differences in the responses of pulp cells to either WMTA or radiopaque dicalcium silicate cements were observed. The results may be partially explained by the fact that a similarity in chemical composition and biocompatibility occurred (Chiang & Ding 2010).

Conclusions

The newly developed radiopaque dicalcium silicate cement with shortened setting time could enhance pulp cell proliferation, differentiation and immunocompatibility. The dental pulp cell responses to radiopaque dicalcium silicate cement was very similar to that reported for WMTA, making it suitable for endodontic use.

Acknowledgements

The authors acknowledge the support of the Chung Shan Medical University Hospital under the project CSH-2011-C-019.

References

Aeinehchi M, Eslami B, Ghanbariha M, Saffar AS (2003) Mineral trioxide aggregate (MTA) and calcium hydroxide as pulp-capping agents in human teeth: a preliminary report. *International Endodontic Journal* **36**, 225–31.

Akira S, Hirano T, Taga T, Kishimoto T (1990) Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB Journal* **4**, 2860–7.

Aubin JE, Liu F, Malaval L, Gupta AK (1995) Osteoblast and chondroblast differentiation. *Bone* **17**, 77S–83S.

Bargholz C (2005) Perforation repair with mineral trioxide aggregate: a modified matrix concept. *International Endodontic Journal* **38**, 59–69.

Camilleri J, Pitt Ford TR (2006) Mineral trioxide aggregate: a review of the constituents and biological properties of the material. *International Endodontic Journal* **39**, 747–54.

Chen CC, Ho CC, Chen David CH, Ding SJ (2009a) Physicochemical properties of calcium silicate cements for endodontic treatment. *Journal of Endodontics* **35**, 1288–91.

Chen CC, Ho CC, Chen David CH, Wang WC, Ding SJ (2009b) In vitro bioactivity and biocompatibility of dicalcium silicate cements for endodontic use. *Journal of Endodontics* **35**, 1554–7.

Chen CL, Kao CT, Ding SJ, Shie MY, Huang TH (2010) Expression of the inflammatory marker cyclooxygenase-2 in dental pulp cells cultured with mineral trioxide aggregate or calcium silicate cements. *Journal of Endodontics* **36**, 456–8.

Chiang TY, Ding SJ (2010) Comparative physicochemical and biocompatible properties of radiopaque dicalcium silicate cement and mineral trioxide aggregate. *Journal of Endodontics* **36**, 1683–7.

Cooper GM (2000) *The Cell: A Molecular Approach*, 2nd edn. Washington, DC: the American Society for Microbiology, Sunderland, MA: Sinauer Associates, pp. 571–607.

Ding SJ, Shie MY, Wang CY (2009) Novel fast-setting calcium silicate bone cements with high bioactivity and enhanced osteogenesis in vitro. *Journal of Materials Chemistry* **19**, 1183–90.

Ding SJ, Kao CT, Chen CL, Shie MY, Huang TH (2010a) Evaluation of human osteosarcoma cell line genotoxicity effects of mineral trioxide aggregate and calcium silicate cements. *Journal of Endodontics* **36**, 1158–62.

Ding SJ, Shie MY, Hoshiba T, Kawazoe K, Chen G, Chang HC (2010b) Osteogenic differentiation and immune response of

human bone marrow-derived mesenchymal stem cells on injectable calcium silicate-based bone grafts. *Tissue Engineering. Part A* **16**, 2343–54.

Felippe WT, Felippe MCS, Rocha MJC (2006) The effect of mineral trioxide aggregate on the apexification and periapical healing of teeth with incomplete root formation. *International Endodontic Journal* **39**, 2–9.

Gomes-Filho JE, Rodrigues G, Watanabe S *et al.* (2009) Evaluation of the tissue reaction to fast endodontic cement (CER) and Angelus MTA. *Journal of Endodontics* **35**, 1377–80.

Gong J, Traganos F, Darzynkiewicz Z (1994) A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. *Analytical Biochemistry* **218**, 314–9.

Gronthos S, Mankani M, Brahim J, Gehron RobeyP, 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**, 13625–30.

Hernandez EP, Botero TM, Mantellini MG, McDonald NJ, Nör JE (2005) Effect of ProRoot® MTA mixed with chlorhexidine on apoptosis and cell cycle of fibroblasts and macrophages in vitro. *International Endodontic Journal* **38**, 137–43.

Islam I, Chng HK, Yap AUJ (2006) X-ray diffraction analysis of mineral trioxide aggregate and Portland cement. *International Endodontic Journal* **39**, 220–5.

Koulaouzidou EA, Papazisis KT, Yiannaki E, Palaghias G, Helvatjoglu-Antoniades M (2009) Effects of dentin bonding agents on the cell cycle of fibroblasts. *Journal of Endodontics* **35**, 275–9.

Minamikawa H, Deyama Y, Nakamura K *et al.* (2009) Effect of mineral trioxide aggregate on rat clonal dental pulp cells: expression of cyclooxygenase-2 mRNA and inflammation-related protein via nuclear factor kappa B signaling system. *Journal of Endodontics* **35**, 843–6.

Moghaddame-Jafari S, Mantellini MG, Botero TM, McDonald NJ, Nör JE (2005) Effect of ProRoot MTA on pulp cell apoptosis and proliferation in vitro. *Journal of Endodontics* **31**, 387–91.

Moretti ABS, Oliveira TM, Sakai VT, Santos CF, Machado MAAM, Abdo RCC (2007) Mineral trioxide aggregate pulpotomy of a primary second molar in a patient with agenesis of the permanent successor. *International Endodontic Journal* **40**, 738–45.

Paranjpe A, Zhang H, Johnson JD (2010) Effects of mineral trioxide aggregate on human dental pulp cells after pulp-capping procedures. *Journal of Endodontics* **36**, 1042–7.

Rezende TM, Vieira LQ, Sobrinho AP, Oliveira RR, Taubman MA, Kawai T (2008) The influence of mineral trioxide aggregate on adaptive immune responses to endodontic pathogens in mice. *Journal of Endodontics* **34**, 1066–71.

Ribeiro DA, Duarte MAH, Matsumoto MA, Marques MEA, Salvadori DMF (2005) Biocompatibility in vitro tests of mineral trioxide aggregate and regular and white Portland cements. *Journal of Endodontics* **31**, 605–7.

Shahi S, Rahimi S, Yavari HR *et al.* (2010) Effect of mineral trioxide aggregates and Portland cements on inflammatory cells. *Journal of Endodontics* **36**, 899–903.

Torabinejad M, White DJ (1995) Tooth filling material and method of use. U.S. Patent No. 5,415,547.

van't Hof RJ, Ralston ST (2001) Nitric oxide and bone. *Immunology* **103**, 255–61.

Vosoughhosseini S, Lotfi M, Shahi S *et al.* (2008) Influence of white versus gray mineral trioxide aggregate on inflammatory cells. *Journal of Endodontics* **34**, 715–7.

Zhang W, Li Z, Peng B (2010) Ex vivo cytotoxicity of a new calcium silicate–based canal filling material. *International Endodontic Journal*, **43**, 769–74.

