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Biodegradable porous calcium polyphosphate scaffolds for the three-dimensional culture of dental pulp cells

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Abstract

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Aim To develop a three-dimensional culture model of human dental pulp cells (DPCs) with biodegradable porous calcium polyphosphate (CPP) scaffolds.

Methodology Human DPCs were isolated from three donors. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to evaluate the cytotoxicity of CPP compared with hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). Values were analysed using unpaired *t*-tests. Cells were seeded onto porous CPP scaffolds with pore sizes in the range of 200–300 μm. The nature of cellular adaptation in the three-dimensional culture model was then evaluated visually by scanning electronic microscopy (SEM) and confocal laser scanning microscopy (CLSM). The apoptotic property of cells on the scaffolds was also assessed by DNA staining with CLSM.

Results The cytotoxicity assay indicated that there was no significant difference between CPP and HA for each donor's original cells ($P > 0.05$). Calcium polyphosphate had no cytotoxic effect on DPCs, whilst SEMs showed that cells successfully adhered to CPP scaffolds and spread amongst pores. On the cell surface, fine processes and matrix secretory granules were found. Confocal laser scanning microscopy showed that cells took on a three-dimensional structure with signs of vitality.

Conclusion Porous CPP scaffolds are promising for the establishment of a three-dimensional culture model of DPCs.

Keywords: cell culture, dental pulp cell, porous calcium polyphosphate, scaffold, three-dimensional.

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Introduction

The dental pulp is a loose connective tissue that not only functions to provide nutritional and sensory properties to dentine, but also has its own reparative capacity. Recent research reports have focused on mechanisms of reparative dentine formation following carious and traumatic dental injury. Dental pulp cells (DPCs) have characters of stem/progenitor cells, which can be used as an *ex vivo* model to elucidate the mechanisms (Gronthos *et al.* 2000, 2002, Goldberg & Smith 2004). Yet, the dental pulp is a tissue with a three-dimensional microenvironment. Therefore, a three-dimensional cell culture model, mimicking the natural environment, might be more useful in pulp biology research and necessary for pulp tissue engineering or for cytotoxicity tests (Schmalz *et al.* 1999, Camps *et al.* 2002).

The materials applied in scaffolds for three-dimensional culture have mostly included the calcium phosphates [i.e. calcium hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and materials composed of HA and

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β-TCP] (Gronthos *et al.* 2000, 2002, Batouli *et al.* 2003). Compared with other calcium phosphate ceramics, HA is inert and the dissolution rate of β-TCP is high. Composite materials have been thus developed to improve the osteoconductivity and biodegradation property. Recently, calcium polyphosphate (CPP) ceramic, characterized by a Ca : P ratio of 0.5 and a linear P–O–P linkage structure, has been proposed as a new scaffold material because of its favourable osteoconductivity and biodegradable property (Lee *et al.* 2001, Park *et al.* 2004). Previous studies have demonstrated the potential of porous CPP scaffolds for anchoring laboratory created articular cartilage (Bhardwaj *et al.* 2001), which in turn can be anchored to bone as a result of bone ingrowth into the porous CPP (Grynpas *et al.* 2002). The interest in porous CPP scaffolds for three-dimensional culture of DPCs lies in its potential application for tissue engineering involving soft and mineralized connective tissues. The purpose of this work was to evaluate the cytotoxicity of CPP on DPCs and to develop an *ex vivo* three-dimensional culture model of DPCs with porous CPP scaffolds.

Materials and methods

Synthesis and preparation of porous CPP scaffolds

$Ca(H_2PO_4)_2{\cdot}H_2O$ (Sigma-Aldrich, St Louis, MO, USA) was thermally treated at 500 °C for 10 h to obtain CPP powders. The powders were melted at 1100 °C with a heat-up rate 15 °C min^{-1} under atmospheric conditions and held for 1 h at the same temperature. The melt was promptly quenched in distilled water to avoid crystallization upon cooling. The amorphous frits were milled and screened to yield powders in a size range of <25 μm. Then CPP was sintered at 875 °C for 1 h. The product was characterized by X-ray diffractometry (XRD) and *in vitro* degradation studies. Porous CPP was fabricated by addition of porous agent mixed with the amorphous powder. The porosity of samples was approximately 70% with interconnected pores in 200–300 μm range (Qiu *et al.* 2005). HA and β-TCP, serving as control materials, were obtained from National Engineering Research Center for Biomaterials, China.

Cell culture

Healthy human impacted third molars were collected from three consenting donors (age 22, 22 and 24 years) at the West China Stomatology Hospital of Sichuan University. Tooth surfaces were swabbed with 75% alcohol and cracked open to reveal the pulp chamber. The pulp tissue was gently separated and minced with scalpels and then digested in a solution of 3 mg mL^{-1} collagenase type I (Sigma-Aldrich) for 50 min at 37 °C. Enzymatically released DPCs were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) (Hyclone, South Logan, UT, USA), 2 mmol L^{-1} L-glutamine, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.2% trypsin and 0.02% ethelenediaminetetraacetic acid (EDTA). Immunocytochemical staining was carried out for tissue origin characterization. Cells between the third and sixth passages were used in the following.

Analysis of cytotoxic effect of CPP on DPCs

Calcium polyphosphate, HA and β-TCP disks were extracted at 37 °C for 72 h in complete medium (DMEM + 10% FCS). The ratio of the disks to the volume of medium was 0.75 cm^2 mL^{-1} as described previously (Nalçacl *et al.* 2004). Cells were seeded into 96-well plates (Corning Inc., New York, NY, USA) at a density of 2×10^4 cells/well. After 24 h attachment, cells were treated with various extracts of materials (200 μL/well) for 72 h. In control wells, equal volumes of complete medium were added. MTT (Sigma-Aldrich) assay was performed to evaluate the cytotoxicity of CPP, compared with HA and β-TCP. In brief, at 4 h after the addition of MTT reagent, the formazan reaction product was extracted with dimethylsulphoxide (Sigma-Aldrich). The optical densities were measured at 570 nm. Six replicates of each material extraction were performed in the MTT assay.

Confocal laser scanning microscopy (CLSM)

Cells were seeded onto porous CPP scaffolds, and then cultured for 14 days. Cells were then fixed with 4% paraformaldehyde in PBS (pH 7.4) and permeabilized with PBS containing 0.1% Triton X-100. Cells were stained with BODYPY-FL phalloidin (Molecular Probes, Eugene, OR, USA) to label the F-actin filaments. Nuclei was counter-stained with 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes), a UV-excited blue-emitting fluorochrome (excitation 358 nm, emission 461 nm). Moreover, the samples were washed again and mounted under glass

coverslips. The fluorescent images were obtained using a confocal laser scanning microscope (MRC1024; Bio-Rad, Hercules, USA) with appropriate filters.

Scanning electron microscopy

Cellular capability of adhesion and growth on porous CPP surface was investigated visually by scanning electron microscopy (SEM). For observation, DPCs were cultured on the CPP scaffolds for 14 days, and fixed in 2.5% glutaraldehyde in PBS at 4 °C for 3 h. Specimens were then washed twice with PBS for 10 min, and then dehydrated by increasing the concentration of alcohol (50, 70, 80, 90, 95, 99 and 2 × 100%). The critical point drying of specimens was undertaken with liquid CO_2. The specimens were sputter-coated with gold and examined by SEM (S-450; Hitachi).

Statistical analysis

All values obtained were analysed using unpaired *t*-tests, with a significance level of $P < 0.05$.

Results

Characterization of CPP

The CPP ceramic obtained was identified as β-CPP phase (JCPDS no. 77–1953) from the XRD pattern (Fig. 1a). Analysis of the surface structure with SEM showed many deposits on the surface of CPP after degradation (Fig. 1c) compared with its original surface (Fig. 1b).

Effect of CPP on viability of DPCs

The MTT values indicated that there was no significant difference between CPP and HA for each donor original cells ($P > 0.05$). CPP slightly increased proliferation of DPCs from donor 2, compared with β-TCP ($P < 0.05$). Figure 2 showed that CPP had no cytotoxic effect on DPCs up to 3 days of culture as did HA or β-TCP, according to standards (1999; ISO10993–5).

Morphology observation of DPCs on porous CPP scaffolds

Confocal laser scanning microscopy showed that DPCs constituted a three-dimensional structure on the scaffolds (Fig. 3). Cellular actin filaments combined with phalloidin displayed green fluorescence. In addition, the cells showed signs of vitality from the staining of nuclear DNA (blue fluorescence), which also suggested that the CPP scaffolds had good biocompatibility. As shown in Fig. 4, CPP scaffolds can improve cell adhesion and migration. DPCs successfully adhered to CPP scaffolds, proliferated and spread among pores

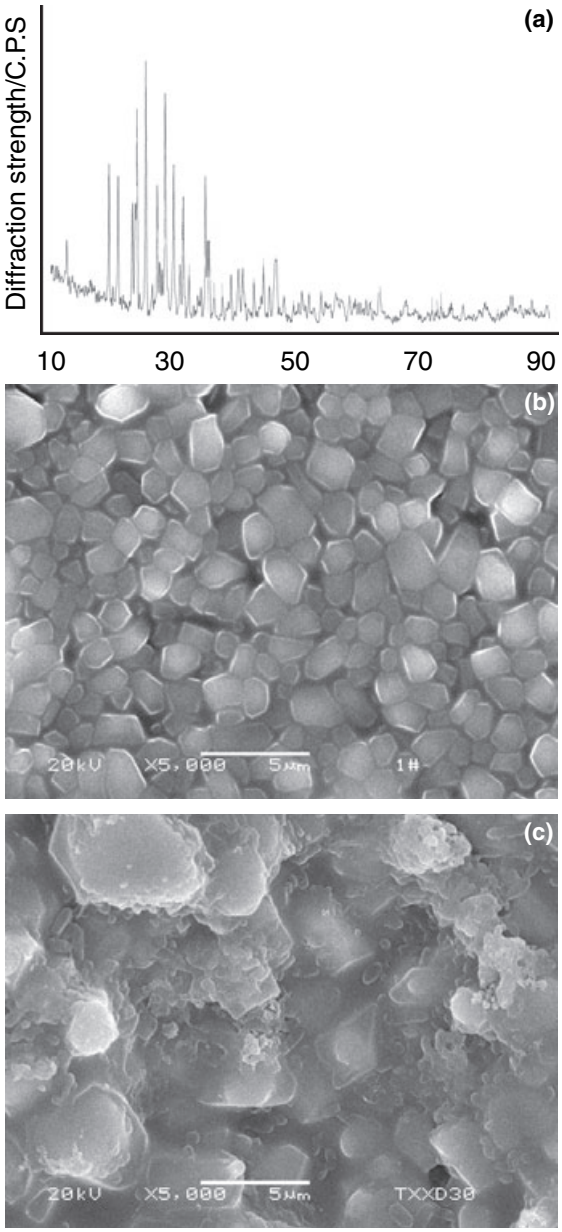

Figure 1 Characterization of calcium polyphosphate (CPP). (a) X-ray diffractometry (XRD) analysis of CPP powder. The surface of original CPP (b) and that of CPP after 20 days degradation (c) was examined by SEM. Magnification: 5000×.

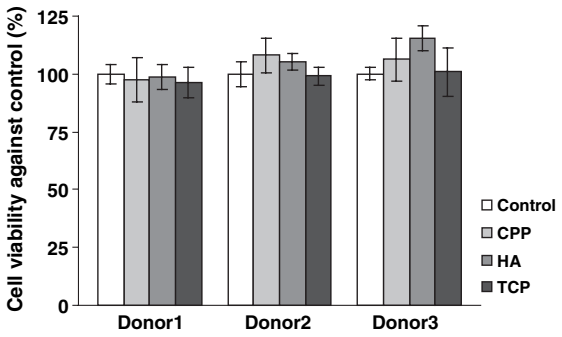

Figure 2 Analysis of the cytotoxic effect of calcium polyphosphate (CPP) on dental pulp cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Percentage of absorbance from each kind of material, compared with that of control was calculated. Data are reported as mean and the bars indicate SD ($n = 6$).

within the three-dimensional network. Interestingly, many fine processes and matrix secretory granules could be seen on cell surface (Fig. 4d, 4f).

Discussion

Tissue engineering from cultured cells is a novel approach to restore dental structure. The scaffold, an essential element in tissue engineering, should provide a physicochemical and biological three-dimensional microenvironment for cell growth and differentiation, promoting cell adhesion and migration (Nakashima & Akamine 2005). In this study, a new scaffold containing inorganic compounds of porous CPP was introduced. During the degradation of CPP, the released PO_4^{3-} and Ca^{2+} resulted in many deposits on the surface. This means the material can offer the two ions to DPCs when CPP are used as scaffolds or pulp capping materials; PO_4^{3-} and Ca^{2+} are vital for calcified tissue formation (e.g. dentine formation). A physicochemical surface is suggested to be prerequisite for odontoblast differentiation (Nakashima & Reddi 2003). In addition, scaffolds such as HA and calcium phosphate are used to enhance bone conductivity (Jadlowiec *et al.* 2003).

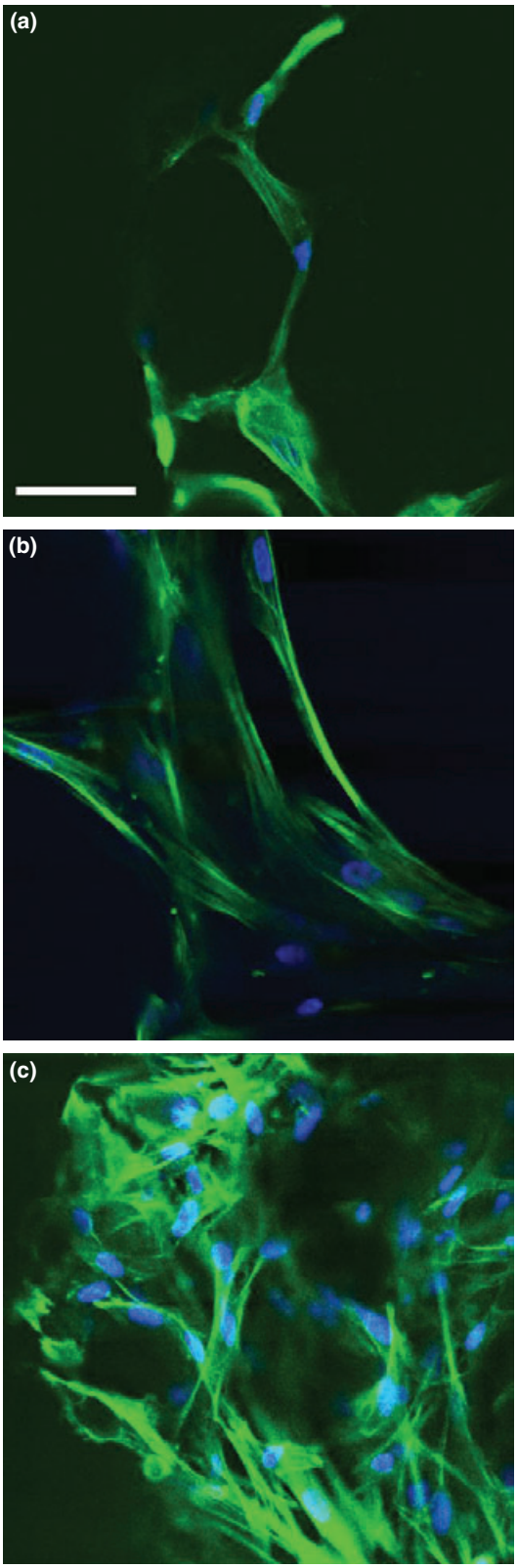

Figure 3 Confocal laser scanning microscopy (CLSM) images of dental pulp cells culture on porous calcium polyphosphate (CPP) scaffolds. (a), (b) and (c) refer to different sites in scaffolds, with cells in diverse densities. The green represents the F-actin filaments and the blue represents the nucleus. Bar, 25 μm. Magnification: 600×.

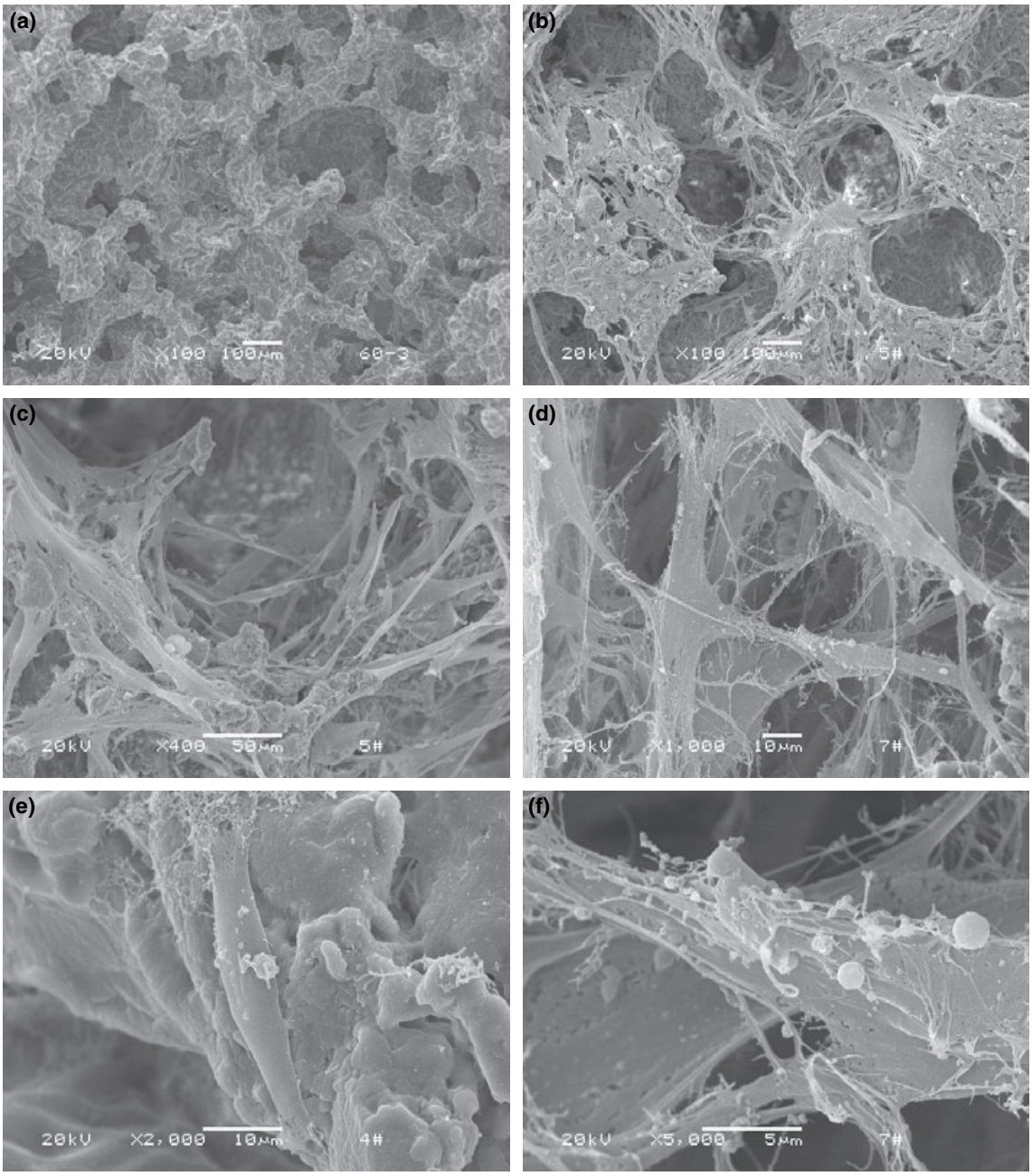

Figure 4 Scanning electron microscopy (SEM) photographs show surface morphology of porous calcium polyphosphate (CPP) scaffold without (a) or with dental pulp cells (b). Cultured cells spread among the pores of CPP scaffold (c, d) and attach to surface of degradable scaffold (e). Matrix forms on cellular surface (f). Different magnifications of SEM photographs are shown: (a) 100×, (b) 100×, (c) 400×, (d) 1000×, (e) 2000× and (f) 5000×.

These data indicates that CPP scaffold is of great promise in future studies.

Biomedical scaffolds should be biocompatible and nontoxic. One of the first steps in the development of a novel scaffold is the evaluation of its cytotoxicity. In the present study, the effect of CPP on cytotoxicity of human DPCs was examined. The cytotoxicity effect of CPP was compared with HA and β-TCP, which are

already being used as biomaterials in humans and animals. Due to the effect of inter-cell-line differences (Wyk *et al.* 2001), the cytotoxicity of CPP on DPCs from three donors was tested. MTT assay revealed that DPCs treated with the CPP extraction did not exert cytotoxic responses, presenting as good a biocompatible property of CPP as HA and β-TCP. Furthermore, signs of vitality of pulp cells demonstrated by DAPI staining confirmed the excellent property of CPP. Previous *ex vivo* studies also showed that CPP was nontoxic on human and rat bone marrow-derived stromal cells (Lee *et al.* 2001, Park *et al.* 2004). Considered together, CPP has no adverse effects on the vitality and proliferation of DPCs.

Dental pulp tissue, with circulation through the narrow root foramen, is surrounded by hard tissues. Once DPCs depart from the natural environment, their biological property may change. In the present study, DPCs were cultured in three-dimensional spaces. The porous scaffolds allowed for effective transport of nutrients, oxygen and metabolic waste. DPCs adhered to CPP scaffolds, proliferated and spread among pores. Cells may interact with each other by cell–cell junctions in spatial arrangement. Additionally, cells can migrate on the scaffolds. The migration of progenitor cells from a reservoir in dental pulp to the defect is essential in that these cells contribute to replacement and regeneration of dentine–pulp complex injury. Furthermore, many fine processes and matrix secretory granules were shown on cell surfaces, with DPCs demonstrating favourable metabolic activity in the porous CPP scaffolds. In previous studies, when DPCs with HA/TCP as scaffolds were implanted into immunocompromised mice, dentine–pulp complex-like structure was formed (Gronthos *et al.* 2000, 2002). Presumably, DPCs seeded onto CPPs scaffolds can also differentiate into functional odontoblast-like cells.

It is uncertain, however, whether the engineered cultures functionally resemble native pulp tissue. How to generate a well defined architecture, which favours cell growth to recreate as closely as possible the *in vivo*-like architecture, is also a key determination in tissue engineering. Actually, the three-dimensional culture models do provide a more physiologically relevant system than classical two-dimensional cell culture models currently utilized for laboratory investigation of pulp biology (Goldberg & Smith 2004) and testing of biocompatibility (Huang & Chang 2002, Nalçacl *et al.* 2004). In particular, the porous CPP scaffolds in the present study, can be readily fabricated with desirable macro- and microstructures. It can be used to form a defined architecture to guide pulp cell growth and development as needed. Moreover, actin cytoskeleton and nucleus were observed with confocal laser scanning microscope. Other staining techniques, i.e. immunofluorescence, may also be feasible. Therefore, it is convenient to study the biological character of DPCs with the three-dimensional culture model. Apart from seed cells and scaffolds, morphogens is also a key element of tissue engineering (Reddi 1998, Nakashima & Reddi 2003). Further progress in CPP scaffolds depends on a three-dimensional microenvironment with optimal delivery of morphogens, which promotes pulp cells behaviour including proliferation, migration and polarization.

Conclusion

In summary, porous CPP scaffold is a beneficial material in the three-dimensional culture of DPCs. Further studies, with the aid of this model, are feasible for elucidating biological characters of DPCs. The current investigations into DPCs using porous CPP scaffolds was a first step towards that long-term goal of biological regenerative endodontic therapy.

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