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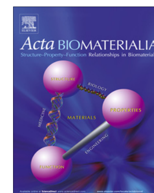

Full length article

Pulp regeneration in a full-length human tooth root using a hierarchical nanofibrous microsphere system

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A B S T R A C T

While pulp regeneration using tissue engineering strategy has been explored for over a decade, successful regeneration of pulp tissues in a full-length human root with a one-end seal that truly simulates clinical endodontic treatment has not been achieved. To address this challenge, we designed and synthesized a unique hierarchical growth factor-loaded nanofibrous microsphere scaffolding system. In this system, vascular endothelial growth factor (VEGF) binds with heparin and is encapsulated in heparin-conjugated gelatin nanospheres, which are further immobilized in the nanofibers of an injectable poly (L-lactic acid) (PLLA) microsphere. This hierarchical microsphere system not only protects the VEGF from denaturation and degradation, but also provides excellent control of its sustained release. In addition, the nanofibrous PLLA microsphere integrates the extracellular matrix-mimicking architecture with a highly porous injectable form, efficiently accommodating dental pulp stem cells (DPSCs) and supporting their proliferation and pulp tissue formation. Our *in vivo* study showed the successful regeneration of pulp-like tissues that fulfilled the entire apical and middle thirds and reached the coronal third of the full-length root canal. In addition, a large number of blood vessels were regenerated throughout the canal. For the first time, our work demonstrates the success of pulp tissue regeneration in a full-length root canal, making it a significant step toward regenerative endodontics.

Statement of Significance

The regeneration of pulp tissues in a full-length tooth root canal has been one of the greatest challenges in the field of regenerative endodontics, and one of the biggest barriers for its clinical application. In this study, we developed a unique approach to tackle this challenge, and for the first time, we successfully regenerated living pulp tissues in a full-length root canal, making it a significant step toward regenerative endodontics.
This study will make positive scientific impact and interest the broad and multidisciplinary readership in the dental biomaterials and craniofacial tissue engineering community.
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1. Introduction

Dental caries, also known as tooth decay, is a major oral health problem all over the world [1–4]. Clinically, if caries progresses and severely inflames the pulp tissues inside the tooth, a root canal procedure is often performed to remove the necrotic dental tissues and seal the pulp chamber with bio-inert materials. While this therapy has been used for many years with high success rates, the repaired tooth is devitalized, becomes brittle and is susceptible to post-operative fracture [5,6]. Therefore, there is a need to develop an alternative method to traditional root canal treatment.

Tissue engineering strategy represents a promising approach to replacing damaged dental structures and restoring their biological functions [7,8]. A number of reports have demonstrated the success of regenerating pulp-like tissues by combining scaffolds and dental stem cells including dental pulp stem cells (DPSCs), stem

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cells from human exfoliated deciduous (SHED), and stem cells of the apical papilla (SCAP) [9–11]. However, pulp regeneration in a full-length root canal has long been a challenge. This is because: (A) The blood supply to the pulp tissues inside the root canal is only from the apical end due to the unique anatomical constraint of a tooth; (B) The full length of a human permanent tooth root is in the range of 11–13 mm [12]; and (C) The apical canal opening of a mature tooth for blood vessel entrance is small (<1 mm), which severely limits nutrient diffusion and the ingrowth of blood vessels, thus affecting the vitality of the implanted cells in the root canal.

Several approaches have been developed and attempted to address this challenge. Blood clotting is a simple technique of revascularization. While this method is used for pulp revascularization of immature permanent teeth in the clinic, the outcome of the treatment is inconsistent, and there are no systematic clinical studies to prove its effectiveness [13]. Angiogenic growth factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and transforming growth factor β (TGF-β), were added to the scaffolds to enhance angiogenesis during pulp regeneration [14–17]. When the growth factor-loaded cell/scaffold constructs were subcutaneously implanted into immunocompromised nude mice for a few weeks, vascularized soft connective tissues similar to dental pulp were observed [16,17]. However, those studies adopted a tooth slice model with a length of 3 mm, which is much shorter than the length of a human permanent tooth root (11–13 mm). Another method is the combined culture of DPSCs and endothelial cells to enhance angiogenesis [18]. The *in vivo* study showed that the DPSC/endothelial cell group exhibited more pulp-like tissues and revascularization than the DPSC group [19]. However, this approach regenerated only pulp-like tissues up to the lower (3.3 mm) to the middle third (5 mm) of the root canal and could not reach the upper regions of the root canal [19]. Recent work reported the injection of SHEDs and a peptide hydrogel into a full-length (11 mm) human root canal and the regeneration of vascularized pulp tissues within the canal [20]. However, both sides of the tooth root were left open in that study, which is not a model to simulate clinical application. To date, vascularized pulp regeneration in a full-length human tooth root with one end sealed has not been achieved.

In this study, we developed an injectable hierarchical microsphere system for full-length pulp regeneration. In this system, the growth factor VEGF binds with heparin and is encapsulated into heparin-conjugated gelatin (HG) nanospheres, which are further immobilized in the nanofibrous biodegradable poly(L-lactic acid) (PLLA) microspheres (MSs) (Fig. 1). The obtained hierarchical microsphere (abbreviated as HG-MS) acts as both a cell carrier and a controlled growth factor delivery vehicle. As a cell carrier, the HG-MS is self-assembled with PLLA nanofibers that mimic the architecture of natural collagen fibers at a nanometer scale. The extracellular matrix-mimicking nanofibrous architecture has been shown to enhance DPSC adhesion, proliferation, differentiation, and pulp tissue regeneration [4,21,22]. As a controlled growth factor delivery vehicle, the heparin-binding VEGF is encapsulated in the HG nanospheres and entrapped by the nanofibers of the PLLA microsphere. VEGF has binding domains with heparin [23], and the binding of VEGF to heparin protects this growth factor from denaturation and proteolytic degradation, which subsequently prolongs its sustained release. Therefore, the release of the VEGF from the HG-MS is controlled by a multiple-layer manner, which includes the binding with the heparin, the degradation of the HG nanosphere, and the physical adsorption of the HG-MS nanofibers. We hypothesized that this hierarchical injectable microsphere scaffolding system would provide an excellent environment to regenerate vascularized pulp tissues in a full-length human tooth root.

2. Materials and methods

2.1. Materials

Gelatin (Type B, from bovine skin, 225 g Bloom, average molecular weight = 50,000, Cat# G9391), and heparin (sodium salt from porcine intestinal mucosa, M_W = 17–19 kDa) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidise (HRP, 304 units/mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Thermo Scientific (Rockford, IL, USA). Poly(vinyl alcohol) (PVA, 88% hydrolyzed, M_W = 88,000), ethyl acetate (EA), dichloromethane (DCM), morpholino ethanesulfonic acid (MES) and N-hydroxy sulfosuccinimide (NHS) were purchased from Acros Organics (New Jersey, USA). Hydrogen peroxide (H_2O_2) aqueous solution (35%, w/w) was obtained from BDH Chemicals (Westchester, PA, USA). Dialysis membrane (molecular weight cut-off (MWCO): 10 kD and 50 kD) was bought from Spectrum Laboratories (Dallas, TX, USA). The human dental pulp stem cells (DPSCs) were a gift from Dr. Songtao Shi, University of Pennsylvania School of Dental Medicine. Human umbilical vein endothelial cells (HUVECs) and endothelial cell growth media kits (EGM-2 BulletKit) were purchased from the Lonza Company (Allendale, NJ, USA). Recombinant human VEGF165 and human VEGF Quantikine ELISA Kits were acquired from R&D Systems, Inc. (Minneapolis, MN, USA).

2.2. Synthesis of HG

The HG was synthesized as we previously reported [24,25]. Briefly, heparin (0.125 g) and gelatin (0.50 g) were dissolved separately in an aqueous solution (12.5 ml) containing 50 mM MES and 0.2 M NaCl. Next, EDC (0.138 g) and NHS (0.033 g) were added to the heparin solution under magnetic stirring for 15 min. The two solutions were mixed and left to react for 12 h at room temperature. The raw HG product was dialyzed for 3 days and freeze-dried. The purified HG was stored in a desiccator for later use. The amount of heparin conjugated to the gelatin was determined by a toluidine blue assay [24,25].

2.3. Encapsulation of HG nanospheres in HG-MS

The HG-MS was prepared using a combination of the water-in-oil-in-oil (W/O/O) double emulsion process, chemical crosslinking, and thermally induced phase separation as described recently by our group [25]. Briefly, HG aqueous solution (2%) with 0.5% PVA was added into EA and emulsified prior to pouring it into a PLLA solution of DCM. Next, glutaraldehyde (20 μl) was added to the W/O emulsion. Under rigorous mechanical stirring, the W/O emulsion was gradually poured into glycerol to form a W/O/O emulsion. Then, the emulsion was quenched in liquid nitrogen to induce phase separation. After 10 min, pre-cooled ethanol (−80 °C) was added for solvent exchange. Glycine (100 mM) was added to neutralize the unreacted glutaraldehyde in the mixture. The HG-MSs were collected, washed with distilled water, and sieved to obtain different ranges of sizes. Finally, the HG-MSs were lyophilized and stored in a desiccator for later use. The encapsulation efficiency of the HG nanospheres in the HG-MS was calculated as described in our previous publication [25].

For comparison, gelatin was used instead of HG during the fabrication process, and the hierarchical microsphere obtained was abbreviated as G-MS. The porosity, diameter and length of the nanofibers, and the apparent density of the microsphere were calculated as we reported previously [26].

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