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Osteogenic differentiation of stem cells from human exfoliated deciduous teeth on poly(ε-caprolactone) nanofibers containing strontium phosphate

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ABSTRACT

Mimicking the architecture of the extracellular matrix is an effective strategy for tissue engineering. Composite nanofibers similar to natural bone structure can be prepared via an electrospinning technique and used in biomedical applications. Stem cells from human exfoliated deciduous teeth (SHEDs) can differentiate into multiple cell lineages, such as cells that are alternative sources of stem cells for tissue engineering. Strontium has important functions in bone remodeling; for example, this element can simulate bone formation and decrease bone resorption. Incorporating strontium phosphate into nanofibers provides a potential material for bone tissue engineering. This study investigated the potential of poly(ε-caprolactone) (PCL) nanofibers coated or blended with strontium phosphate for the osteogenic differentiation of SHEDs. Cellular morphology and MTT assay revealed that nanofibers effectively support cellular attachment, spreading, and proliferation. Strontium-loaded PCL nanofibers exhibited higher expressions of collagen type I, alkaline phosphatase, biomineralization, and bone-related genes than pure PCL nanofibers during the osteogenic differentiation of SHEDs. This study demonstrated that strontium can be an effective inducer of osteogenesis for SHEDs. Understanding the function of bioceramics (such as strontium) is useful in designing and developing strategies for bone tissue engineering.

1. Introduction

Engineering bones for reconstructive surgery requires a suitable cell source, optimal signals for cell functioning, and a biodegradable scaffold to serve as a temporary extracellular matrix (ECM). The ECM has numerous functions, such as providing support for cell growth, segregating tissues, and regulating intercellular communication and other dynamic cellular processes. Natural bone structure is composed of highly organized fibrillar proteins, such as collagen type I, for the deposition of crystalline minerals. Thus, mimicking the structure of the ECM is an effective strategy to design and develop appropriate implanting scaffolds for bone tissue engineering.

Nanofibers, which are composed of ultrafine and continuous fibers, have recently attracted attention in regenerative medicine engineering because of their high porosity, variable pore-size distribution, high surface-to-volume ratio, and capacity to imitate the ECM structure for tissue engineering. Electrospinning uses an electric field to convert a polymer solution into continuous polymer fibers, and had been applied to fabricate fibrous scaffolds that mimic the structure of the natural ECM. The resulting fibers with diameters ranging from microns to a few nanometers have a nonwoven structure.

Polymeric nanofibrous scaffolds have positive effects on bone regeneration [1,2]. Inorganic bioactive materials, such as tricalcium phosphate, biphasic calcium phosphate, and hydroxyapatite (HA), have been applied to bone tissue engineering by electrospinning polymer/bioactive material solutions into composite nanofibers. The other method is by mineralizing nanofibers through direct coating of bioactive materials on the surface via immersion in simulated body fluid [3–6]. These complexes improve bone behavior and exhibit better alkaline phosphatase (ALP) expression and osteocalcin deposition.

Strontium traces found in calcified tissue are crucial in mineralizing bone tissues and dental caries [7]. Strontium is present in the mineral phase of bones, particularly in regions of high metabolism in new bones [8]. Bone cement-containing strontium has good bioactivity and strong bone-binding capacity [9]. Previous studies have shown that strontium can induce prostaglandin production and cyclooxygenase expression to increase osteoblastic differentiation of human mesenchymal stem cells (MSCs) and rat bone marrow-derived MSCs [10,11]. The beneficial effects of strontium on bone reconstruction are closely related to its capability to increase bone formation and decrease bone resorption [12,13].

Stem cells from human exfoliated deciduous teeth (SHEDs) have recently attracted attention as novel multi-potential stem cell sources [14,

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15]. The loss of primary teeth creates the perfect opportunity to recover and store this convenient source of stem cells. Isolating SHEDs is simple, painless and convenient, and involves little or no trauma. These immature stem cells are important in the regeneration and repair of craniofacial defects, tooth loss, and bones because of their capability to proliferate and differentiate [16]. SHEDs generate rapidly and grow faster than adult stem cells, thus suggesting that they are less mature. SHEDs are postnatal stem cells capable of differentiating into osteogenic, odontogenic, adipogenic, and neural cells [17].

In this study, poly(ε-caprolactone) (PCL) nanofiber scaffolds containing strontium were used to evaluate the osteogenic differentiation of MSCs. The objective of this study was to blend or coat nanofibers with strontium phosphate while maintaining their fibrous and porous structure to evaluate the osteogenic differentiation potential of SHEDs.

2. Materials and methods

2.1. Isolation and culture of SHEDs

SHEDs were collected from seven-year-old children ($n = 3$), and the cells were isolated according to the method described by Miura et al. [17]. The pulps were separated gently from the crown and then digested in 3 mg/mL type I collagenase and 4 mg/mL dispase for 1 h at 37 °C. A single cell was suspended in alpha-modified Eagle's medium (α-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μM L-ascorbic acid 2-phosphate, and 20 ng/mL basic fibroblast growth factor for ordinary culture.

2.2. Fluorescence-activated cell sorting (FACS)

SHEDs were suspended in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin at a concentration of 5.82×10^6 cells/mL. The cells were then incubated for 30 min on ice with FITC-conjugated anti-human CD45, phycoerythrin (PE)-conjugated anti-human CD105, FITC-conjugated anti-human CD34, PE-conjugated anti-human CD73, PE-conjugated anti-human CD90, Oct-4, and stage-specific embryonic antigen 4 (SSEA4) [Becton, Dickinson, and Company (BD), USA]. Analyses were performed with a BD FACSCalibur flow cytometer (BD, USA) using WinMDI software (Scripps Research Institute, USA).

2.3. Preparation of PCL nanofibers containing strontium phosphate

Strontium phosphate was synthesized by mixing 0.8 M $SrCl_2$ with 0.2 M Na_2HPO_4 for 1 d agitation, then rinsed with distilled water fully and dried in the oven. The composition analysis of strontium phosphate was measured by EDX. PCL nanofibers were fabricated using an electrospinning system composed of a syringe pump, a syringe, Teflon™ fluidic tubing, an 18-gauge blunt-tip catheter, and an adapter. A high-voltage power source was connected to the catheter tip with a standard alligator clamp. The collector is a piece of aluminum foil with an electrical tape. Then, 14% PCL (80,000 Mw, Sigma-Aldrich Corporation, USA) and 0.9% strontium phosphate were dissolved in a mixed solution with 1:1 formic acid:acetone volume ratio. PCL solutions with and without strontium phosphate were placed in a 10 mL syringe connected to an 18-gauge needle with a tip-to-collector distance of 23 cm. The solution was fed into the needle by a syringe pump at a rate of 2 mL/h, and a voltage of 18 kV was applied to generate a polymer jet. Strontium phosphate was mineralized on pure PCL nanofibers through the soaking method. The PCL nanofibers were immersed in a strontium phosphate solution and stored at room temperature for 10 h during the coating process.

2.4. Measurement of contact angle and mechanical properties

The water contact angle of the nanofibrous surface was measured by the sessile drop method with a G10 contact angle goniometer at room temperature (First Ten Angstroms 1000 Drop Share Instrument B Frame System). A water droplet was placed on the fiber surface, and the contact angle was measured after 10 s. Tensile measurement was performed on the fibers using a universal testing machine (PT-003, Kotsao,Taiwan). The tested sample was cut into a thin strip, and a tensile test was conducted at a crosshead speed of 50 mm/min at room temperature.

2.5. Cellular morphology observation and cell proliferation assay

The morphology of the cells in the scaffold was examined using a scanning electron microscope (SEM, FEI Quanta 200, Philips, Netherlands). SHEDs grown on PCL nanofibers with and without strontium phosphate were fixed with 4% paraformaldehyde for 30 min at room temperature. The samples were rinsed with PBS, dehydrated in sequentially increasing ethanol solutions to 100 vol.%, and then dried by CO_2 Pelco critical-point dryer #2400 (Ted Pella Inc., USA). The specimens were sputter-coated with a thin layer of gold and then examined under SEM.

MTT assay was used to measure the metabolic activity of mitochondrial enzymes and to estimate cellular proliferation on the PCL nanofibers. Tetrazolium salts were transformed using cellular mitochondrial dehydrogenase into visible dark blue formazan deposits such that the amount of color produced was directly proportional to the number of viable cells. The entire scaffold that contains the proliferated cells was treated with 5 mg/mL MTT at 37 °C for 24 h. The cell culture medium was removed, and formazan was dissolved in dimethyl sulfoxide. The metabolized MTT was evaluated in terms of its optical density in a spectrophotometer (Multiskan™ FC, Thermo Scientific™, USA) at 540 nm.

2.6. Osteogenic differentiation

SHEDs from passages 15 to 22 were seeded into the nanofibers at 2×10^4 cells/scaffold with the proliferated medium to evaluate the effects of SHEDs cultured in nanofibers with induced strontium on bone differentiation. After 2 d of culture, differentiation was processed in an osteogenic medium (α-MEM, Gibco®, Life Technologies™, USA) containing 10% heat-inactivated FBS, 20 μg/mL ascorbic acid, 8 mM β-glycerol phosphate, 10^{-8} M dexamethasone, and 1% penicillin/streptomycin solution. The medium was replaced every 3 d for 21 d.

2.7. Real-time reverse transcription (RT)-polymerase chain reaction (PCR)

The total RNA from SHEDs was extracted using TRIzol reagent (Ambion®, Life Technologies™, USA). The quantity and purity of the RNA were determined at an absorbance of 260/280 nm. First-strand cDNA was synthesized from 500 ng of the RNA by using Super Script® III Reverse Transcriptase kit (Life Technologies™, USA), and real-time PCRs were performed following Invitrogen™ Corporation (USA) protocols. RT-PCR was performed using Smart Quant Green Master Mix with dUTP and ROX according to Protech Technology Enterprise Co., Ltd. (Taiwan) protocols. Forty-five cycles of quantitative-RT-PCR were performed for the target genes and the housekeeper gene GAPDH. The

Table 1
The sequences of primers used for the real time PCR.

Gene name	Primer sequences
Type I collagen	F: 5′-TGCTTGAATGTGCTGATGACAGGG-3′ R: 5′-TCCCCTCACCCTCCCAGTAT-3′
Osteonectin	F: 5′-AGGTATCTGTGGGAGCTAATC-3′ R: 5′-ATTGCTGCACACCTTCTC-3′
Osteocalcin	F: 5′-ACCACATCGGCTTTCAG-3′ R: 5′-CAAGGGCAAGGGGAAGA-3′
GAPDH	F: 5′-ATGAGAAGTATGACAACAGCC-3′ R: 5′-AGTCCTTCCACGATACCAA-3′