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# Osteoblastic differentiation of stem cells from human exfoliated deciduous teeth induced by thermosensitive hydrogels with strontium phosphate

Wen-Ta Su [a,*], Wei-Ling Chou [a], Chih-Ming Chou [b]

[a] Department of Chemical Engineering and Biotechnology National Taipei University of Technology, Taipei, Taiwan
[b] Department of Biochemistry, Taipei Medical University, Taipei, Taiwan

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ABSTRACT

Stem cells from human exfoliated deciduous teeth (SHEDs) are a novel source of multi-potential stem cells for tissue engineering because of their potential to differentiate into multiple cell lineages. Strontium exhibits an important function in bone remodeling because it can simulate bone formation and decrease bone resorption. Hydrogels can mimic the natural cellular environment. The association of hydrogels with cell viability is determined using biological tests, including rheological experiments. In this study, osteogenic differentiation was investigated through SHED encapsulation in hydrogels containing strontium phosphate. Results of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and proliferating cell nuclear antigen (PCNA) immunofluorescence staining indicated that the cells grew well and SHEDs proliferated in the hydrogels. Strontium-loaded chitosan-based hydrogels induced the biomineralization and high expression of alkaline phosphatase. Moreover, the expression levels of bone-related genes, including type-I collagen, Runx2, osteopontin (OP), and osteonectin (ON), were up-regulated during the osteogenic differentiation of SHEDs. This study demonstrated that strontium can be an effective inducer of osteogenesis for SHEDs. Elucidating the function of bioceramics (such as strontium) is useful in designing and developing strategies for bone tissue engineering.
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## 1. Introduction

Clinical therapy is an innovative and effective method that combines biomaterials and cells to form scaffolds for tissue engineering. Thermosensitive hydrogels comprise low-viscosity hydrophilic polymeric aqueous solution at room temperature and can undergo gel transition at increased temperature. Hydrogel can absorb water up to several folds of its dry weight and thus provide a 3D network. This property allows cell attachment, proliferation, and differentiation in hydrogels.

Chitosan (GS) is an amino-polysaccharide and a natural biodegradable cationic polymer that has recently attracted much interest in studies and clinical applications; chitosan exhibits excellent biocompatibility, biodegradability, anti-microbial activity, wound healing ability, low immunogenicity, low cost, cell growth promotion, and osteo-conduction properties [1–3]. Chitosan is soluble in acidic solution because of the electrostatic repulsion among the polymer chains. At pH higher than 6.2, neutralization of the structural chains of chitosan induces the formation of gel-like aggregation. β-Glycerophosphate (β-GP) is the most commonly used gelling agent in thermo-induced gelation mechanism; β-GP is a simple phosphate donor and a potent inhibitor of phosphatase. This compound promotes bone matrix mineralization when delivered to osteoblasts because it functions as a source of phosphate ions. Gelatin (G) has been widely examined as a tissue engineering scaffold because it demonstrates high biocompatibility and biodegradability, as well as identical composition and almost similar biological properties to collagen. Gelatin also contains many integrin-binding sites for cell adhesion and differentiation [4].

Bone regeneration through cell-based therapies requires stem cells to differentiate into osteoblasts and osteocytes. Embryonic stem cells and induced pluripotent stem cells can differentiate into cells characterized by three layers in vitro and in vivo; these cells are also useful for cell-based therapy [5,6]. Bone marrow-derived mesenchymal stem cells (MSCs) are most widely used for in vitro investigation of osteoblast differentiation; MSCs derived from umbilical cord and adipose tissues can also differentiate into osteoblasts [7,8]. Stem cells from human exfoliated deciduous teeth (SHEDs) have recently attracted attention as novel multi-potential stem cell sources [9]. Isolation of SHED is simple, straightforward, and convenient and causes minimal or no trauma. Every child loses his or her primary teeth; thus, this convenient source of stem cells can be easily recovered and stored. Immature stem cells can extensively proliferate and differentiate, and thus function as an important source of stem cells for the regeneration and repair of craniofacial defects, tooth loss, and bones [10]. SHEDs rapidly generate and grow faster than adult stem cells; hence, these cells are less mature and can develop into various tissues in a process called transdifferentiation or

* Corresponding author at: 1 Sec. 3, Chung-Hsiao E. Rd, Taipei 10608, Taiwan.
*E-mail address:* f10549@ntut.edu.tw (W.-T. Su).

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plasticity. SHEDs are a population of postnatal stem cells that can differentiate into osteogenic, odontogenic, adipogenic, and neural cells [11].

Strontium positively affects the osteogenic differentiation of bone marrow MSCs [12–14], which exist at the implant site and are characterized by an intrinsic osteogenic potential. SHEDs are a potential candidate to renew degenerating tissues and restore tissue functions because of their multi-potential differentiation. An anti-osteoporotic drug called strontium ranelate is composed of an organic moiety with two stable strontium atoms. Pre-clinical studies have shown that strontium ranelate dissociates bone resorption from formation, thereby increasing bone mass and strength. Strontium-containing bone cement also demonstrates good bioactivity and bone-binding strength [15]. Previous studies have shown that strontium can induce prostaglandin production and cyclooxygenase expression to increase the osteoblastic differentiation of MSCs derived from humans and rat bone marrow [16]. The beneficial effects of strontium on enhancing bone formation are closely related to its capability to increase bone formation and decrease bone resorption [17]. Strontium can increase bone mass by activating osteoblast activity and suppressing osteoclast function. Our previous studies have confirmed that strontium-substituted bioactive scaffolds can induce osteogenic differentiation from SHEDs and increase alkaline phosphatase (ALP) activity and calcium mineralization [18].

The present study aimed to investigate the effects of chitosan-based hydrogels containing strontium phosphate on the osteogenic differentiation of SHEDs in vitro. The development of osteogenesis was determined by analyzing the gene expression of osteoblastic markers, ALP activity, and matrix mineralization.

## 2. Materials and methods

### 2.1. Isolation of SHEDs and fluorescence-activated cell sorting (FACS) analysis

Human exfoliated deciduous molars were obtained as discarded biological samples from three different seven-year-old children at the Dental Clinic of the Kaohsiung Medical University with informed consent and followed the approved Institutional Review Board (IRB) guidelines, and were isolated according to the method from our previous study [19]. To confirm the attribution of isolated cells, SHEDs were suspended in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin at a concentration of $5.8 \times 10^6$ cells/mL. The cells were then incubated for 30 min on ice with FITC-conjugated anti-human CD45, PE-conjugated anti-human CD105, FITC-conjugated anti-human CD34, PE-conjugated anti-human CD73, and PE-conjugated anti-human CD90 (BD Biosciences, USA). Analyses were performed with BD-FACSCalibur flow cytometer (Becton Dickinson) using the WinMDI software.

### 2.2. Preparation of thermosensitive gel

A total of 2.5 wt.% chitosan (GS) and 1 wt.% gelatin (G) were dissolved in 0.1 M acetic acid with or without strontium phosphate or tricalcium phosphate (Sigma). A synthesis of strontium phosphate was previously described in detail in our research [19]. Briefly, 1 g of β-GP was dissolved in deionized water and then sterilized with an autoclave to prepare 50 wt.% β-GP solution. The β-GP solution was added dropwise to the CS/G solution under stirring, and pH was adjusted to 7.4. Finally, the mixed solutions were incubated at 37 °C for gelation. The released strontium concentration from CS/G/β-GP/Sr hydrogel in PBS was measured by inductively coupled plasma-optical emission spectrometer (ICP, Optima 8300).

### 2.3. Morphological observation

The structural feature of lyophilized hydrogel was observed through scanning electron microscopy. Pore size was determined through imaging analysis using the photos of sectional samples by scanning electron microscope (SEM, FEI Quanta 200, Philips, Netherlands).

### 2.4. Rheological measurement

The gelation of solution was measured with a rheometer (Anton Paar MCR302). The viscoelastic properties of gels were assessed by measuring the storage and loss moduli; the former represents the elastic behavior of the gels and the latter reflects the viscous behavior. Storage modulus G  and loss modulus G  were measured versus temperature at a gap of 0.1 mm and at a frequency of 1 Hz. The temperature at the cross point of G  and G  was defined as the gelation temperature.

### 2.5. Hydrogel degradation

All hydrogel samples were added into phosphate buffer saline (PBS, pH 7.4) and then incubated at 37 °C for 21 d. The samples were removed from the medium at tested time points and then weighed. The degree of in vitro degradation was expressed as weight loss: weight loss $= (Wo - Wt) / Wo \times 100\%$, where $Wo$ is the original weight of the hydrogel before degradation and $Wt$ is the weight of the hydrogel at the tested time.

### 2.6. Cellular viability assay

An indirect 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay measures the metabolic activity of mitochondrial enzymes. This assay was performed to estimate cellular viability in the hydrogel containing induced supplements. Tetrazolium salts were transformed using cellular mitochondrial dehydrogenase into visible dark blue formazan deposits. The amount of color produced was directly proportional to the number of viable cells. The whole hydrogel containing proliferated cells was treated with 5 mg/mL MTT at 37 °C for 24 h. The cell culture medium was removed, and formazan was solubilized in DMSO. The optical density of the metabolized MTT was determined using a spectrophotometer at 540 nm (Thermo Scientific Multiskan FC).

### 2.7. ALP assay

An ALP assay was performed. The differentiated cells in the hydrogels were first rinsed with PBS to remove the remaining culture medium. Approximately 500 μL of PBS containing 0.1 M glycine, 1 mM $MgCl_2$, and 0.5% Triton X-100 (pH 10.5) were added to each sample to rupture the cell membranes and release the ALP molecules. The samples were then incubated for 1 h. About 100 μL of the supernatant was extracted and transferred into microcentrifuge tubes wrapped in aluminum foil. Briefly, 200 μL of p-nitrophenyl phosphate solution (Sigma) was added to each tube. The microcentrifuge tubes were then placed in a water bath at 37 °C for 30 min and then transferred in an ice bath at 0 °C for 10 min to reduce the reaction rate. About 50 μL of 3 N NaOH solution was added to each tube to terminate the reaction. The collected solutions were placed into a 96-well microplate. The plate was subjected to an ELISA reader (Thermo Scientific Multiskan FC) at a wavelength of 405 nm. The ALP test was performed in triplicates.

### 2.8. Calcium quantification

The differentiated cells in the hydrogels were subjected to calcium quantification on 7, 14, and 21 d of culture. The accumulated calcium in the secreted mineral matrix of the osteoblasts was quantified via Alizarin red S staining. The samples were washed twice with PBS and then immersed in 95% ethanol solution for 30 min. The samples were stained with 1% Alizarin red S for 10 min, washed three times with PBS, and solubilized with 10% cetylpyridinum chloride. The calcified nodules appeared as red and were observed through light microscopy. Total

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