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Strontium (Sr) elicits odontogenic differentiation of human dental pulp stem cells (hDPSCs): A therapeutic role for Sr in dentine repair?

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ABSTRACT

Strontium (Sr) forms a significant component of dental restorative materials and although it is widely used in toothpastes, the biological effects of Sr on the dentine-pulp complex have not been investigated. In this first study, we characterise the Sr elicited effects on human dental pulp stem cells (hDPSC) *in vitro* using exogenously Sr added to culture medium, and bioavailable Sr derived from a novel bioactive glass (BG). The related mechanisms were also investigated. Our results indicate that low dose Sr (between 0.1 and 2.5 mM) induces proliferation and alkaline phosphatase (ALP) activity of hDPSCs, but has no effect on colony formation or cell migration. Sr at specific concentrations (1 and 2.5 mM) stimulated collagen formation and mineralisation of the hDPSC generated matrix. In addition, qRT-PCR, Western blotting and immunocytochemistry revealed that Sr regulates gene expression and the protein secretion of the odontogenic markers: dentine sialophosphoprotein (DSPP) and dentine matrix protein 1 (DMP-1) and protein localisation (DSPP was localised to the Golgi, while no apparent changes occur in DMP-1 distribution which remains in both cytosol and the nucleus). Additionally, the calcium sensing receptor (CaSR) and downstream pathway MAPK/ERK signalling pathway in hDPSCs were activated by Sr. Bioavailable Sr from the BG revealed novel biological insights of regulating metabolic and ALP activities in hDPSCs. Taken together, these results suggest that Sr at specific doses significantly influences proliferation, odontogenic differentiation and mineralisation of hDPSCs *in vitro* via the CaSR using a pathway with similarities to osteoblast differentiation. These are the first such studies and indicate that Sr treatment of hDPSCs could be a promising therapeutic agent in dental applications. In conclusion, we propose that Sr from a substituted BG could be used more effectively in biomaterials designed for dental applications.

Statement of Significance

Despite the fact that strontium (Sr) is used widely in dental practise, its potential effects on odontoblasts have been ignored. Our study provides the first evidence that Sr (exogenous and that derived from a bioglass (BG)) can stimulate dentinogenesis in human dental pulp stem cells (hDPSCs) by promoting their proliferation, differentiation and mineralisation *in vitro*. Therefore, while previously unrecognised, Sr BG is likely to be beneficial in atraumatic dentistry practise and maintenance of a competent tooth in conditions such as caries. Repair of defected dentine is still one of the main challenges in dental research and annually untreated caries results in the loss of productivity equivalent to US\$ 27 billion. Advances in tissue engineering technology, alongside the use of dental pulp stem cells provide an approach to achieve dentine regeneration. Understanding the actions of Sr will permit a more controlled application of Sr in the clinic. These data are thus likely to be of great interest to the material scientists, biological researchers, clinicians and manufacturers of dental products.

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1. Introduction

Dentine is a major constituent of teeth and it protects the dental pulp – which, in turn, primarily provides nutrition and acts as a biosensor to detect potential pathogenic stimuli [1]. Carious

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defects in dentine and loss of enamel may affect the pulp viability with subsequent decreases in tooth strength and increased fragility. Preservation of the dentine mass is therefore crucial for maintaining the whole tooth.

Annually, untreated caries results in the loss of productivity equivalent to US\$ 25.14 billion for permanent teeth, and US\$2.09 billion for deciduous teeth [2]. Recent advancement in tissue engineering technology provides an approach to achieve dentine regeneration by replacement or repair of the impaired dentine-pulp tissues [3]. There are three key factors for optimal tooth tissue engineering: growth factors, suitable biomaterials and responsive stem/progenitor cells [4]. A unique population of postnatal human dental pulp stem cells (hDPSCs) from human adult dental pulp tissue have been characterised by Gronthos et al. [5]. These cells demonstrate characteristic stem cell properties, and also show rapid proliferative rate, as well as the capacity to form mineralised dentine-like tissue both *in vivo* and *in vitro* [5,6]. Due to their regenerative potential, the use of hDPSCs in dental regeneration is favoured [7–9]. A range of materials have been studied in the regeneration of hard dental tissues, including hydrogel scaffolds [10], degradable synthetic polymers [11], bioceramics [12], as well as mineral trioxide aggregate (MTA) [13]. However, while these materials have beneficial effects, none are perfect; therefore new biomaterials that possess more appropriate properties and bioactivity remain to be identified.

Strontium (Sr), in trace amounts, is a normal constituent of tooth structure. However, following capping of dental pulp in dogs using strontium hydroxide ($\text{Sr}(\text{OH})_2$), Sr was identified in the dental pulp [14,15]. As a result of its low systemic toxicity and high atomic number Sr-based materials are widely used in dental materials in the clinic to locate the restorations by X-ray. Additionally, Sr is added as an active agent of dentifrices for treating dentine hypersensitivity. Originally, strontium chloride (SrCl_2) was used in dentifrices but more recently strontium acetate ($\text{Sr}(\text{C}_2\text{H}_3\text{O}_2)_2$) at a loading of 8% w/w has been used. Sr is also used as a component of many dental restoratives to repair decayed teeth, particularly in glass ionomer cements, such as Fuji IX[®] (GC, Japan) which has the composition: 12.9%Al – 22.5% Si – 1.7%P – 0%Ca – 12.6%F – 5.6%Sr [16]. In these cases, Sr is in close association with exposed dentinal tubules. Occlusion of these tubules, and with high external Sr concentration, there is the potential for Sr to traverse internally and reach the pulp cavity. Given the widespread dental use of Sr, it is surprising that so little is known regarding its biological activity on odontoblasts.

It is well known that Sr can alter pre-osteoblast/osteoblast behaviour to induce mineralised bone-like nodules [17]. Recently, Strontium ranelate ($\text{C}_{12}\text{H}_6\text{N}_2\text{O}_8\text{SSr}_2$, at 1–2 g/day) has been approved for osteoporosis therapy. The related molecular mechanisms by which Sr regulates osteoblasts, include the calcium sensing receptor (CaSR) and CaSR-downstream pathway, such as mitogen-activated protein kinase (MAPK) signalling pathway [18] and Wnt/ β -catenin signalling pathway [19]. Osteoblasts and odontoblasts both produce an extracellular matrix protein scaffold which subsequently mineralises [20]. As Sr can influence pre-osteoblast/osteoblast behaviour [21] and mesenchymal stem cells (MSC) differentiation [22,23], the hypothesis is that Sr would influence hDPSCs behaviour. Additionally, identifying molecular mechanisms involved in Sr mediated odontogenic differentiation of hDPSCs will substantiate the use of Sr in dentine pulp tissue engineering. Previously it has been demonstrated that Sr substituted for calcium (Ca) in bioactive glass (BG), increased osteoblast proliferation, differentiation as well as inhibiting osteoclast-mediated bone resorption [24–27]. Hence we sought to investigate the effects of Sr alone and Sr substituted BG conditioned medium (BGCM) on the odontogenic differentiation of hDPSCs, mineralising potential and related molecular mechanisms.

2. Materials and methods

2.1. Cell culture

Human dental pulp stem cells (hDPSCs, Lonza, Switzerland) were cultured in the Dulbecco's Modified Eagle's medium (DMEM, Lonza, Switzerland) supplemented with 10% fetal bovine serum (FBS) and antibiotics (10 U/L penicillin and 100 mg/L streptomycin) in a humidified atmosphere containing 10% CO_2 at 37 °C with medium change every two days.

Passages 3–5 were used for all experiments. Cells were seeded in 96-well plates (1000 cells/well) for proliferation assay and ALP activity assay, or in 12-wells plates (1×10^5 cells/well) for the scratch assay. 1×10^5 cells/dish or 1×10^3 cells/dish in 10 cm dishes for Western blot and colony formation respectively. 5×10^3 cells were also seeded on to sterilised cover slips and placed in 12-wells plates for immunocytochemistry. For Alizarin Red S and Sirius red staining, 24-wells plates were used with 5×10^3 cells/well cultured for 2, 3, and 4 weeks. Cells treated with odontogenic medium (OM, containing 10% FBS, 50 $\mu\text{g}/\text{ml}$ L-ascorbic acid, 5 mM β -glycerophosphate and 10nM dexamethasone) was used as positive control for odontogenic differentiation and mineralisation study.

Sr was added to the medium of the confluent cultures at various concentrations as the chloride compound $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$. The medium was then subjected to inductively coupled plasma-optical emission spectrometry (ICP-OES), to confirm the available concentrations of Sr in the medium (Fig. S1).

2.2. Cell proliferation assay

Total DNA content was measured to quantify the cellular proliferation as previously described [28]. Briefly, the plates were collected on 1, 4, 7, and 10 days after Sr (0, 0.5, 2.5, 5, 10 mM) treatment, the cells washed twice with PBS and stored at -20°C . With all time points collected, cells were thawed at room temperature and 100 μl distilled water was added to each well, incubated for 1 h and then refrozen. After 24 h they were thawed again and 100 μl of the fluorochrome Hoechst 33258 at the concentration 20 $\mu\text{g}/\text{ml}$ in high salt TNE buffer (2 M NaCl) was added to each well. The plates were read with excitation at λ 350 nm and emission at λ 460 nm. Cell number was calculated according to the cell number standard curve (Fig. S2A). The results were collected from three independent experiments.

2.3. Quantitative assay of ALP activity

To determine the early Sr induced differentiation of hDPSCs, ALP activity was assessed following exposure to Sr as in the cell proliferation assay. Plates were collected and washed in PBS and stored at -20°C . Once all plates were collected, cells were thawed and 100 μl ALP reaction solution (20 mg 4-Nitrophenyl-phosphate disodium salt hexahydrate tablet was dissolved in 8 ml Tris buffer solution (pH = 9.5) containing 15 μl of 2 M MgCl_2) was added to cell lysate, incubated in 37 °C for 1 h. The resultant coloured reaction product, pNP, was measured at 405 nm with a spectrophotometer. ALP activity was calculated according to the standard curve (Fig. S2B) and normalized to cell number. Experiments were performed three times.

2.4. Colony formation assay

After incubation for 14 days, cells were washed with PBS twice, fixed with formalin for 15 min, and stained with 0.5% crystal violet for 15 min at room temperature. The colony is defined to consist of

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