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Zinc bioglasses regulate mineralization in human dental pulp stem cells

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ARTICLE INFO

Article history:
Received 17 September 2016
Received in revised form
6 January 2017
Accepted 9 March 2017

Keywords:
Zinc
Bioglass
Dental pulp stem cells
Mineralization
DSPP
DMP-1
VEGF

ABSTRACT

Objective. A promising strategy in regenerative endodontics is the combination of human dental pulp stem cells (hDPSCs) with an appropriate biomaterial substrate. The effects of zinc and zinc containing bioactive glasses (ZnBGs) on hDPSCs have been characterized in this study.

Methods. ZnBGs were designed and produced. Then the odontogenic differentiation and mineralization potential of hDPSCs upon ZnBGs treatment were investigated.

Results. Free Zn ions (0–5 ppm) enhanced proliferation and alkaline phosphatase (ALP) activity of hDPSCs. Further, ZnBGs conditioned medium (ZnBG-CM) increased the production and secretion of odontogenic markers: dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP-1). In addition, we identified that mRNA expression of the osteogenic markers *RUNX2, OCN, BSP, BMP-2, MEPE* and *ON* was increased following treatment with ZnBG-CM. Long term treatment with ZnBG-CM increases the formation rate of mineralized nodules (similar to hydroxyapatite, Ca:P = 1.6), as confirmed by scanning electron microscopy combined with energy dispersive X-ray spectroscopy (SEM-EDX). Lastly, the administration of ZnBG-CM induces VEGF expression.

Significance. These findings implicate that ZnBG would be beneficial in regenerative endodontics and could influence the way present Zn containing clinical products are used.

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1. Introduction

The high incidence of tooth decay and tooth loss combined with the limited regeneration capacities of dental tissues necessitates the development of novel strategies for dental practices [1]. Human dental pulp stem cells (hDPSCs) are a mesenchymal stem cell (MSC) population [2,3]. Analogous to MSCs derived from bone marrow (BMSCs), hDPSCs can form alizarin red-positive mineralized deposits, physiologically similar to hydroxyapatite (HA) [2,4]. Thus, experiments have been conducted to evaluate the value of hDPSCs in the repair of bone defects [5–7]. However, numerous studies have demonstrated that hDPSCs have a greater ability to differentiate into odontoblasts compared to BMSCs [2,8]. Co-transplanting cultured hDSPCs with HA/TCP particles, into immunocompromized mice, led to the vascularisation of a

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http://dx.doi.org/10.1016/j.dental.2017.03.011
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fibrous pulp tissue surrounded by a layer of odontoblast-like cells, cells had extended tubular structures that resembling a dentin-like structure [2,8]. In contrast, a lamellar bone structure with associated adipose tissue was formed by human BMSCs under the same experimental condition [2]. These findings clearly demonstrate distinct tissue regeneration capacities of hDPSCs and BMSCs, which appear to have maintained a level of epigenetic memory of their tissue origin [2]. Somewhat unsurprisingly then, hDPSCs is the preferred derivation of cells for dental tissue engineering.

The fundamental concept in hard tissue engineering lies in combining a scaffold with living cells to repair damaged tissue [9]. Scaffolds provide a suitable environment for cell attachment, proliferation, differentiation and to promote biomineralization [10]. A significant challenge regarding scaffold usage is the choice of a suitable material. A broad range of materials, natural or synthetic polymers and inorganics, have been designed as scaffolds for dentin-pulp regeneration [11]. Bioactive glasses (BGs) are a promising choice for repair and reconstruction of hard tissues, such as bone and dentin [12,13]. BGs can form hydroxyapatite (HA) quickly, is biocompatible and can deliver 'therapeutic ions' [12,14].

In dental practice, the use of zinc (Zn) is mostly favored owing to its bacteriostatic and cariostatic properties [15]. For example, Zn oxide eugenol (ZOE), Zn oxyphosphate (ZOP), and Zn polycarboxylate (ZPC) are widely used. However, despite the clinical advantages, the dentinogenic benefits of Zn have yet to be determined. Accordingly, we have focused on Zn and Zn containing BGs (ZnBGs) to characterize their effects on hDPSCs.

2. Materials and methods

2.1. *Bioactive glass synthesis and characterization*

ZnBGs (compositions are shown in Fig. 2A) were synthesized by the melt-quench route [16]. Glass frits were grounded and sieved to powders ≤38 μm. The particle size and amorphous nature was confirmed by master particle size analyzer and X-ray diffraction (XRD). ZnBGs powders were characterized as described previously [17]. Briefly, 75 mg of BGs powder (≤38 μm) was added to 50 ml Tris buffer and incubated on an orbital shaker (60 rpm at 37 °C). Post shaking, the pH values were recorded. The solution was filtered, diluted by a factor of ten in distilled H_2O and acidified with 69% nitric acid (final concentration 2%) prior to determining the elemental concentrations of calcium (Ca), phosphorus (P), silica (Si), and zinc (Zn) using an inductively coupled plasma-optical emission spectrometer (ICP-OES). The residual powders were dried overnight and analyzed to evaluate the extent of apatite formation using XRD, and Fourier transform infrared spectroscopy (FTIR).

2.2. *Cell culture*

Human dental pulp stem cells (hDPSCs, Lonza) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza) supplemented with 10% fetal calf serum (FCS) and antibiotics (10 U/L penicillin and 100 mg/L streptomycin) in a humidified atmosphere with 10% CO_2 at 37 °C.

Free ionic Zn (0.5, 1, 5, 10 and 20 ppm) was added as $ZnCl_2$. ZnBG conditioned medium (ZnBG-CM) was prepared by immersing 75 mg of glass powders in 50 ml DMEM for set periods of time. Before being added to the cells, ZnBG-CM was filter-sterilized, serum and antibiotics were added and equilibrated to 37 °C, 10% CO_2 in an incubator.

Cells between passages 3–5 were used in all experiments. For cell proliferation, MTT and ALP activity assays, 1000 cells/well were seeded in 96-well plates and cultured for 1, 4, 7 and 10 days. 1×10^5 cells/dish and 2×10^4 cells/well were placed in 10 cm dishes and 6-well plate for Western blot and qPCR studies, respectively. 5×10^3 cells were seeded onto sterilized coverslips and placed in 12-well plates for immunocytochemistry. 24-wells plate with 5×10^3 cells/well was used for the mineralization study.

2.3. *MTT activity assay*

The MTT activity assay reflects mitochondrial activity—an indication of viable cells. After rinsing twice with PBS, 50 μl of MTT solution (5 mg/ml MTT in PBS) was added to cell culture and incubated for 4 h at 37 °C. After this, medium was removed and the formazan was solubilized in 100 μl dimethyl sulfoxide (DMSO). The absorbance of the solubilized formazan supernatant was measured at 560 nm.

2.4. *Quantitative assay of ALP activity*

Alkaline phosphatase (ALP) activity was measured by adding 100 μl of ALP reaction solution (20 mg 4-nitropheyl-phosphate disodium salt hexahydrate tablet dissolved in 8 ml Tris buffer solution (pH = 9.5) containing 15 μl of 2 M $MgCl_2$) to cell lysate, incubated in the dark at 37 °C for 1 h. Afterwards, the reaction was stopped by adding 50 μl of 1 M NaOH. The absorbance of the supernatant was measured at 405 nm and ALP activity was calculated according to a standard curve (Fig. S1).

2.5. *Crystal violet staining*

Cells were fixed with 10% neutral buffered formalin for 15 min, rinsed twice with PBS and stained in 0.5% (w/v) crystal violet for 15 min at room temperature. Before visualizing, the unbound stains were removed by extensive washing in PBS.

2.6. *Western blot analysis*

Cell pellets were re-suspended in RIPA buffer (containing 1% Triton X-100 and 1% cocktail proteinase inhibitors) and the protein content measured using DC™ Protein Assay. Intracellular expression of dentin sialophosphoprotein (DSPP), dentin matrix acidic phosphoprotein 1 (DMP-1) and vascular endothelial growth factor (VEGF) was evaluated by Western blotting using indicated antibodies (Tables S1) on a PVDF membrane. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the loading control. Cell culture supernatant was also collected and analyzed for DSPP and DMP-1 secretion,

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