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Three-dimensional zinc incorporated borosilicate bioactive glass scaffolds for rodent critical-sized calvarial defects repair and regeneration

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ABSTRACT

The biomaterials with high osteogenic ability are being intensively investigated. In this study, we evaluated the bioactivity and osteogenesis of BG-Zn scaffolds in vitro and in vivo with a rodent calvarial defects model. Zinc containing borosilicate bioactive glass was prepared by doping glass with 1.5, 5 and 10 wt.% ZnO (denoted as BG-1.5Zn, BG-5Zn and BG-10Zn, respectively). When immersed in simulated body fluid, dopant ZnO retarded the degradation process, but did not affect the formation of hydroxyapatite (HA) after long-period soaking. BG-Zn scaffolds showed controlled release of Zn ions into the medium for over 8 weeks. Human bone marrow derived stem cells (hBMSCs) attached well on the BG-1.5Zn and BG-5Zn scaffolds, which exhibited no cytotoxicity to hBMSCs. In addition, the alkaline phosphatase activity of the hBMSCs increased with increasing dopant amount in the glass, while the BG-10Zn group showed over-dose of Zn. Furthermore, when implanted in rat calvarial defects for 8 weeks, the BG-5Zn scaffolds showed a significantly better capacity to regenerate bone tissue compared to the non-doping scaffolds. Generally, these results showed the BG-Zn scaffolds with high osteogenic capacity will be promising candidates using in bone tissue repair and regeneration.

1. Introduction

In spite of rapid development in biomaterials and biotechnology, there is still a major challenge for the treatment of critical-sized bone defects [1]. To address this issue, new bioactive materials have been fabricated via incorporation of biological essential elements, such as calcium, phosphorus, copper and zinc [2–5], which can enhance bone formation and mineralization meanwhile promote the osseointegration process [4,6].

Zinc is known to play an important role in bone metabolism [7]. Previous studies have indicated that Zn possesses stimulatory effects on bone formation, ability to promote the expression and maintenance of osteoblastic phenotypes in vitro [8–10]. The effects are particularly obvious for Zn containing materials including bone cements [11], coatings [12] and bioactive glasses [13,14]. On the other hand, Zn is a highly selective inhibitor of osteoclastic bone resorption in vitro [15]. Incorporation of Zn into bioactive materials has shown an enhancement of the proliferation and osteogenic differentiation of osteoblast, endothelial and neuronal cells [4,16]. Furthermore, there are some cellular and molecular evidences that incorporation of Zn into biomaterials could up-regulate the expression of osteoblastic relative genes such as alkaline phosphatase (ALP), collagen type I (Col-I), osteocalcin (OCN), and osteopontin (OPN). Up-regulation of these genes could further promote extracellular matrix mineralization by increasing collagen secretion synthesis and calcium deposition [17,18].

Bioactive materials such as 45S5 bioglass and some other composition based on silicate or phosphate systems have been widely used in bone tissue engineering [19–21]. The excellent bone-bonding ability arises from the high rate of formation of hydroxyapatite (HA) at the surface of the material after reaction with the surrounding biological fluids [20]. In addition, previous studies have shown that the ionic products of bioactive glass can

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stimulate gene expression and proliferation of human osteoblasts [22,23]. Recently, it has been reported that borosilicate bioactive glasses had controllable HA conversion rate and offered an attractive delivery system to release inorganic ions for healing bone defects [24,25]. Studies have also shown that the borate glass could stimulate rapid bone formation in rat tibial defects and rat calvarial defects [26,27].

Despite the established roles of Zn in bone metabolism, the feasibility of Zn containing biomaterials in clinical applications still relies on many factors, especially safety issues associated with the zinc content. On the other hand, the effect of Zn on the degradation and bioactivity of borosilicate bioactive glass in simulated body fluid (SBF) and the response of human bone marrow derived stem cells (hBMSCs) has not been studied, and no study on the properties of Zn-doped borosilicate bioactive glass in vitro or in vivo has yet been reported.

In this study, it was hypothesized that Zn-doped borosilicate bioactive glass scaffolds could stimulate osteogenesis, which would be of great interest for applications in bone tissue engineering. The objective of the present study was to create porous 3-dimensional (3D) scaffolds with borosilicate bioactive glasses doped with varying amounts of Zn (1.5, 5 and 10 wt.% ZnO), and to evaluate the effects of dopant Zn on degradation and bioactivity of borosilicate bioactive glass. In vitro response of hBMSCs to the BG-Zn scaffolds was also evaluated. Furthermore, the influence of introduction of Zn into glass on the ontogenesis in osseous defects was systematically investigated using rodent calvarial defects model in vivo.

2. Materials and experiments

2.1. Preparation of BG-Zn scaffolds

The bioactive glass scaffolds were created using a foam replication method as described in detail previously [28]. The parent bioactive glass (designated BG) had a borosilicate composition ($6Na_2O$, $8K_2O$, 8MgO, 22CaO, $36B_2O_3$, $18SiO_2$, $2P_2O_5$; mol%). The glasses composed of BG and BG doped with 1.5, 5 and 10 wt.% ZnO (denoted as BG-1.5Zn; BG-5Zn, and BG-10Zn, respectively) were prepared using conventional melting and casting techniques. Briefly, a mixture of the requisite amounts of analytical grade $ZnCO_3{\cdot}2Zn(OH)_2{\cdot}H_2O$, Na_2CO_3, K_2CO_3, $CaCO_3$, H_3BO_3, SiO_2, $(MgCO_3)_4{\cdot}Mg(OH){\cdot}25H_2O$ and $NaH_2PO_4{\cdot}2H_2O$ (Sinopharm Chemical Reagent Co., Ltd., China) was heated for 1 h at 1200 °C to form a molten glass. The glass frit was ground and sieved to obtain particles of average size ~50 μm. Then a slurry was prepared by mixing 52.6 wt.% bioactive glass particles, 3.5 wt.% ethyl cellulose (analytical grade, Sinopharm Chemical Reagent Co., Ltd., China) and 43.9 wt.% anhydrous ethanol. A polyurethane foam (50 pores per inch) was coated by immersing in the slurry. After removal from the slurry, the coated foam was dried for 8 h in air at room temperature (RT) and heated for 2 h at 450 °C (heating rate = 1.5 °C/min) and then for 2 h at 550 °C (heating rate = 2.5 °C/min) to sinter the glass particles into a dense 3D network.

2.2. Characterization of the degradation and bioactivity of BG-Zn scaffolds

The porosity of the as-prepared scaffolds was measured according to Archimedes' principle. Degradation and conversion of the as-fabricated scaffolds were evaluated as a function of immersion time in SBF, which was prepared according to Kokubo's method [29]. The weight loss of the scaffolds was measured as a function of time and used as a measure of HA conversion of the glass as described previously [30]. A ratio of 1 g of scaffold to 100 ml of SBF was used in all of the conversion experiments. The concentration of released ions in SBF solution was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES; Optima 2100 DV; USA). The fresh immersion solution will be changed at each time point after ICP test. Three samples for each group were used in the immersion test. After the immersion, these samples were mixed together by 1:1:1 (v/v/v), then tested by ICP.

The reacted scaffolds were washed with deionized water, then with ethanol, dried, coated with gold, and examined with a field-emission scanning electron microscope (FESEM, Quanta 200 FEG). Some scaffolds were also embedded in poly(methyl methacrylate) (PMMA), ground to form a flat surface, coated with gold, and examined with a FESEM (Hitachi S-4700; Tokyo, Japan) equipped with an energy-dispersive X-ray (EDX) spectrometer (Apollo X; EDAX, Inc.). EDX analysis was used to examine compositional changes in the scaffolds due to the bioactive glass conversion. The immersed scaffolds were crushed into glass powder for the XRD analysis. XRD was performed at a scanning rate of $1^\circ\ min^{-1}$ in the range of 10–80°.

2.3. In vitro cellular evaluations of BG-Zn scaffolds

2.3.1. Attachment and morphology of hBMSCs on BG and BG-Zn scaffolds

The human bone marrow derived stem cells (hBMSCs) used were supplied by the Sixth People's Hospital, Shanghai Jiao Tong University School of Medicine. The scaffolds were sterilized by heating for 2 h at 180 °C in a dry atmosphere. After sterilization, the four scaffold groups were seeded with 10^5 hBMSCs and cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO; Invitrogen Pty Ltd., Australia) supplemented with 10% fetal calf serum (FCS) (In Vitro Technologies, Australia) in 24-well culture plates at 37 °C in a humidified atmosphere of 5% CO_2. After 2-day culturing, scaffolds with hBMSCs were washed with phosphate buffered saline (PBS) and placed in 2.5% glutaraldehyde in PBS at 4 °C for 1 h. Then, these fixed samples were rinsed with PBS, post-fixed in 1% osmium tetroxide in PBS, dehydrated with a graded series of ethanol, then air dried. The morphology of the attached hBMSCs on BG and BG-Zn scaffolds was examined using FESEM.

2.3.2. Proliferation and ALP activity of hBMSCs cultured on BG and BG-Zn scaffolds

A cell viability assay was used to evaluate the proliferation of the hBMSCs incubated on the fabricated scaffolds (Cell Counting Kit-8 (CCK-8); Dojindo Molecular Technologies, Inc., Japan). 10^4 hBMSCs were cultured ($n = 6$) in BG and BG-Zn scaffolds using the procedure described above for 1, 3 and 7 days. Subsequently, 360 μl of culture medium and 40 μl of CCK-8 solution (9:1) were added to each well at each time point and the system was incubated at 37 °C for 4 h. Aliquots (100 μl) were taken from each well and transferred to a fresh 96-well plate. The absorbance of the samples was measured at 450 nm with a spectrophotometric microplate reader (Bio-Rad 680, USA). The results were expressed as the optical density of the aliquots minus the absorbance of the blank wells.

The differentiation of hBMSCs toward the osteogenic lineage was ultimately demonstrated seeding 10^5 cells ($n = 6$) on each scaffold. The alkaline phosphatase (ALP) activity was measured at 7 and 14 days. After culturing, the medium was decanted, and the scaffolds were rinsed with PBS and 50 mM Tris buffer, then lysed in 200 μl 0.2% Triton X-100. Lysates were sonicated after being centrifuged at 15,000 rpm for 20 min at 4 °C. Finally, 50 μl supernatant was mixed with 150 μl working solution according to the manufacturer's protocol (Beyotime, China). The results were measured at 405 nm by a plate reader (Bio-Rad 680, USA). The ALP activity was calculated from a standard curve after normalizing to the total protein content and the results were expressed in millimoles of *p*-nitrophenol produced per minute per milligram of protein.