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Effects of silica and calcium levels in nanobioglass ceramic particles on osteoblast proliferation

A. Moorthi [1], P.R. Parihar, S. Saravanan, M. Vairamani, N. Selvamurugan *

Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur 603 203, Tamil Nadu, India

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ABSTRACT

At nanoscale, bioglass ceramic (nBGC) particles containing calcium oxide (lime), silica and phosphorus pentoxide promote osteoblast proliferation. However, the role of varied amounts of calcium and silica present in nBGC particles on osteoblast proliferation is not yet completely known. Hence, the current work was aimed at synthesizing two different nBGC particles with varied amounts of calcium oxide and silica, nBGC-1: SiO_2:CaO:P_2O_5; mol% ~ 70:25:5 and nBGC-2: SiO_2:CaO:P_2O_5; mol% ~ 64:31:5, and investigating their role on osteoblast proliferation. The synthesized nBGC particles were characterized by transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDS), Fourier transform infrared (FTIR) spectroscopy and X-ray diffraction (XRD) studies. They exhibited their size at nanoscale and were non-toxic to human osteoblastic cells (MG-63). The nBGC-2 particles were found to have more effect on stimulation of osteoblast proliferation and promoted entering of more cells into G2/M cell cycle phase compared to nBGC-1 particles. There was a differential expression of cyclin proteins in MG-63 cells by nBGC-1 and nBGC-2 treatments, and the expression of cyclin B1 and E proteins was found to be more by nBGC-2 treatment. Thus, these results provide us a new insight in understanding the design of various nBGC particles by altering their ionic constituents with desirable biological properties thereby supporting bone augmentation.

1. Introduction

Superseding the current strategy of using autografts and allografts is one of the major goals of current biomaterial based bone regeneration. Despite various materials emerging, the gap between complete understanding of the material nature and cell interaction is not yet completely understood. The use of various composites for supporting bone formation has been exploited to hunt for a best combination of biomaterials [1–6]. Ceramics have been largely implicated as bone filler materials owing to their bioactivity, chemical similarity to the inorganic phase of bone and ability of osteointegration [7,8]. Substitutions with metals have uplifted the properties of these materials for bone tissue engineering [9,10]. Poor mechanical strength and faster dissolution rates are the major drawbacks encountered with the use of ceramics alone. Hence, they have been used in combination with various polymers as scaffolds for bone tissue engineering [11,12].

Bioglass ceramics (BGCs) with compositions based on calcium oxide, silica or phosphorus pentoxide phosphate systems denote another important group of inorganic and bioactive biomaterials for bone tissue engineering applications. They could elicit a specific biological in vivo response at the interface and attach to the tissues, such as soft tissue and bone, with a strong chemical bond, due to this reason, BGCs have been used for many different applications in bone tissue regeneration [13]. They exhibit unique properties like showing osteoinductive behavior, ability to bond to soft tissue as well as to hard tissue and to form a carbonated hydroxyapatite layer (HCA) when exposed to biological fluid [14,15]. BGCs containing CaO–SiO_2–P_2O_5 have no local or systemic toxicity effect. No inflammation or foreign-body response could be observed using BGCs [16]. By controlling the structural and particle size in nanorange some properties of BGCs such as osteoconductivity, sintering characters, solubility and mechanical reliability can be improved [17]. Studies have demonstrated their enhanced in vitro and in vivo osteoblast functions (e.g. adhesion, proliferation, synthesis of bone-related proteins and deposition of calcium-containing mineral) on nanostructured metals, ceramics, polymers, and composites [18]. It has been shown during the last decade that the cellular response of osteoblasts to BGCs is controlled genetically [19]. It has been observed that ionic dissolution products from BGCs [e.g. Si, Ca, P] and from other silicate based glasses stimulate expression of several genes of osteoblastic cells. Since Ca, Si and P are the main components of biological apatite, the inorganic phase of human bone, these ions play an essential role in bone formation and resorption. For determining the stimulatory effect of these ions on osteoblast proliferation, it is important to know their concentrations and the mechanism of their interaction with bone

* Corresponding author. +91 9940632335 (cell).
E-mail addresses: selvamurugan.n@ktr.srmuniv.ac.in, selvamn2@yahoo.com (N. Selvamurugan).
[1] Present address: Department of Medical Bionanotechnology, Chettinad Academy of Research and Education, Chennai 603 103, Tamil Nadu, India.

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cells. Lower (2–4 mmol) and medium (6–8 mmol) Ca concentrations are suitable for osteoblast proliferation, differentiation and extracellular matrix (ECM) mineralization; whereas higher Ca concentrations (>10 mmol) are cytotoxic [20,21]. The extracellular Ca plays an important role in bone remodeling by directly activating intracellular mechanisms by affecting Ca-sensing receptors in osteoblastic cells. Si is known to be an essential element for metabolic processes associated with the formation and calcification of bone tissue. High Si contents have been detected in early stages of bone matrix calcification, whereby aqueous Si was shown to be able to induce precipitation of hydroxyapatite, the inorganic phase of human bone [22]. So, the present study was aimed to determine the molecular actions on osteoblast proliferation by altering the Ca and Si levels in nanobioglass ceramic (nBGC) particles.

2. Materials and methods

2.1. Materials

Calcium nitrate, tetraethyl orthosilicate (TEOS), polyethylene glycol (Mw: 20,000), and ammonia water were purchased from Sigma Aldrich, USA. All other chemicals used were of reagent grade. Dulbecco's modified Eagle's medium (DMEM) was purchased from Sigma Aldrich, USA. Antibodies were purchased from Santa Cruz Biotechnology, USA. Fetal bovine serum and cell culture reagents were purchased from Invitrogen, USA.

2.2. Methods

2.2.1. Synthesis of nBGC particles

The nBGC particles were synthesized via sol–gel route as described earlier [23]. Two different nBGC particles were synthesized and described as nBGC-1 (SiO_2:CaO:P_2O_5; mol% ~ 70:25:5) and nBGC-2 (SiO_2:CaO:P_2O_5; mol% ~ 64:31:5). The method for synthesizing two different nBGC particles was similar but varies only in the amount of corresponding precursors to calcium oxide and silica. In brief, preweighed calcium nitrate was mixed with calculated amount of tetraethyl orthosilicate (TEOS) and dispersed into the solution of ethanol:water (1:2 ratio) and during this period, the pH of the solution was maintained around 2 by the addition of 1 M citric acid to obtain a clear solution (Solution 1). Separately, diammonium hydrogen orthophosphate was mixed with 2% of poly ethylene glycol (PEG; Mw 20000) and the pH was maintained to 10 by adding ammoniated water (Solution 2). Under continuous refluxing conditions both solutions 1 and 2 were added. The resultant mixture was aged for 24 h at room temperature to obtain a semitransparent white gel precipitate. The precipitate was filtered, washed with deionized water, dried under vacuum and lyophilized and calcined at 700 °C (1 °C/min) for 6 h to obtain white nBGC particles. Phosphorous pentoxide was unaltered. The composition is briefly described in Table 1.

2.2.2. Physico-chemical characterization of nBGC particles

The morphology, size and energy dispersive spectra of nBGC particles were assessed by TEM analysis. nBGC particles were dispersed in suitable solvent and subjected for size and EDS measurements using JEOL-JEM2100F. The spectra of nBGC particles were recorded with a FT-IR spectrophotometer [American Perkin Elmer Co] using KBr press. The spectra were collected over the range of 4000–450 cm^{-1}. The XRD patterns of nBGC particles were obtained at room temperature using a Panalytical XPERTPRO powder diffractometer (CuKα radiation) operating at a voltage of 40 kV. Diffraction patterns were recorded in the range of 0–90° and scanned at a speed of 2° min^{-1}. The inductive coupled plasma atomic emission spectroscopy (ICP-AES) analysis was performed to establish the concentration of calcium ion released by 0.1 mg/ml of nBGC-1 and nBGC-2 in the medium for 24 h, 48 h, 72 h and 96 h using an Optima 2000DV ICP-emission spectrometer.

Table 1
The molar percentage of silica, calcium oxide and phosphorus pentoxide in the nBGC-1 and nBGC-2 particles and the precursors used to prepare these particles.

nBGC types	mol%	$Ca(NO_3)_2.4H_2O$	TEOS	$NH_4H_2PO_4$
	SiO_2:CaO:P_2O_5	(g)	(g)	(g)
nBGC-1	70:25:5	4.150	14.760	0.669
nBGC-2	64:31:5	5.150	13.496	0.669

2.2.3. Cell culture

Human osteoblastic cells (MG-63) were procured from the National Centre for Cell Sciences (NCCS) Pune, India. The cells were grown to confluence in DMEM supplemented with 10% fetal bovine serum (FBS). Then the cells were trypsinized and subcultured for further treatments.

2.2.4. Cytotoxicity evaluation

Cytotoxicity study was carried out using MTT [3-(4, 5-Dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium] to detect the non-toxic concentration of the nBGC-1 and nBGC-2 particles. The nBGC particles were treated to MG-63 cells for 24 h and 48 h. The procedure was followed as described earlier [23]. The OD (Optical density) measurements were recorded, plotted and compared with control. The experiments were performed in triplicates at each time point.

2.2.5. Cell count and viability

Cell count and cell viability were performed using MUSE™ Cell Analyzer. MG-63 cells were seeded in 6 well plates, allowed to adhere onto the plates for an overnight incubation. Cells were serum starved in 0.1% FBS/DMEM medium for a period of 6 h and then treated with 0.1 mg/ml of corresponding nBGC particles for 24 h and 48 h. Cells in the control wells received no treatment instead the medium was changed with DMEM containing 0.1% FBS. At each time point, cells were harvested and subjected for cell count and viability assay according to the manufacturer's protocol (Muse™, Millipore, USA).

2.2.6. Cell cycle analysis

Muse™ cell analyzer was employed in studying the cell cycle analysis. Cells were grown and treated as mentioned above. The cells were then harvested at the end of each treatment period and washed multiple times with 1× PBS (phosphate buffered saline) to remove the debris. Care was taken to the cell suspension that aggregation of the cells was minimized. Finally, cells were resuspended in 50 μl of 1× PBS and fixed in pre-chilled 70% ethanol and stored at −20 °C for 3 h. At the end of fixation, 250 μl of cell suspension was washed with 1× PBS and cell pellet was collected under centrifugation. 200 μl of the Muse cell cycle reagent was added to the pellet and resuspended. After 30 min incubation in dark, the suspension was analyzed for cell cycle phase analysis.

2.2.7. Western blot

At the indicated time point, cells were harvested and whole cell lysates were prepared as described earlier [24]. The proteins were resolved onto 12% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membrane (PVDF). The membranes were blocked in 5% nonfat dry milk solution in 1× TBS (Tris-buffered saline) containing 0.1% Tween-20 and incubated with primary antibody for overnight incubation at 4 °C. After sufficient washing, the membrane was incubated for an hour with secondary antibody conjugated with HRP (horseradish peroxidase). The membrane was washed to remove unbound secondary antibodies and finally the immunoreactive signals from the protein of interest were detected using enhanced chemiluminescence detection kit (Thermo Scientific, USA) and quantified by Image J software. The results were normalized with α-tubulin which served as a loading control.