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Effect of apatite formation of biphasic calcium phosphate ceramic (BCP) on osteoblastogenesis using simulated body fluid (SBF) with or without bovine serum albumin (BSA)

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ABSTRACT

Although biphasic calcium phosphate ceramic (BCP) holds promise in therapy of bone defect, surface mineralization prior to implantation may improve the bioactivity to better integrate with the host. Immersion in simulated body fluid (SBF) and bovine serum albumin-simulated body fluid (BSA-SBF) are common methods to form apatite interface layer. This study was intended to investigate the effect of SBF and BSA-SBF treatment on the bioactivity of BCP *in vitro*. In this study, osteoblasts were grown on BCP with or without treatment of SBF or BSA-SBF, and detected with general observation, scanning electron microscope (SEM), cell proliferation assay, morphology observation, viability assay, alkaline phosphatase (ALP) activity assay, and osteogenic specific gene expression of alkaline phosphatase (*ALPL*), bone gamma-carboxyglutamate (gla) protein (*BGLAP*), bone morphogenetic protein 2 (*BMP2*), bone sialoprotein (*BSP*), type I collagen (*COLI*) and runt-related transcription factor 2 (*RUNX2*) after culture of 2, 5 and 8 days. As the results shown, BCP pre-incubated in SBF and BSA-SBF up-regulated ALP activity and osteogenic related genes and proteins, which testified the positive effect of SBF and BSA-SBF. Especially, BSA-SBF enhanced the cell growth significantly. This study indicated that treatment by BSA-SBF is of importance for BCP before clinical application.

1. Introduction

Calcium phosphates have been widely used for skeletal repair because of biocompatibility, safety, unlimited availability, high cost-effectiveness ratio and mimicking to the mineral phase of bone [1–2]. Among the calcium phosphates, biphasic calcium phosphate ceramic (BCP) attracted most attention as it has advantage over single-phased ceramics by presenting favorable bioactivity and controllable biodegradation [3–4]. Besides, it can be used as vehicle for therapeutic agents or biological factors [5].

After implanted into bone defect, the biomaterials are expected to well integrate with the surrounding bone without forming fibrous tissue interface layer. Since the 1970s, it has been found that several materials integrate with their host bony tissue through formation of an apatite interface layer [6]. It can promote osteogenic cellular activities, mineral deposition and bone formation. Thus, surface mineralization of BCP to form apatite layer may facilitate bone growth. But surface mineralization similar to natural way is a time-consuming process that takes a few weeks, during which partial degradation and release character of encapsulated agents would occur.

Simulated body fluid (SBF) or SBF containing protein molecules (BSA-SBF) has been applied to generate mineralized layer. Immersion in SBF can also be used to measure the ability of biomaterials in formation of apatite layer to predict the bioactivity *in vivo* [7]. This may enhance the bioactivity of ceramics. However, the SBF or BSA-SBF measurement was suspected the indicator of bioactivity of ceramics because of the differences between the actual *in vivo* process of biomaterial integration inside a living human body and the process of measuring apatite forming ability of biomaterials inside SBF solution [8]. Therefore, whether SBF or BSA-SBF treatment on ceramics has the ability to enhance bioactivity [9] was uncertain to us.

In this study, the impact of SBF and BSA-SBF immersion on bioactivity of BCP was investigated by comparison of osteoblast genesis of BCP with or without treatment of SBF or BSA-SBF. Examination of the cell proliferation, morphology, viability and alkaline phosphatase (ALP)

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activity was performed. Further, osteogenic specific gene expression was also studied.

2. Materials and methods

2.1. Simulated body fluid (SBF) and bovine serum albumin (BSA) treatment

Biphasic calcium phosphate ceramic (BCP, HA/β-TCP = 60/40, porosity >75%) was purchased from Engineering Research Center in Biomaterials of Sichuan University (Sichuan, China), which was prepared as disc. The method for *in vitro* SBF immersion of ceramic has been described by Mcon K et al. [10]. Briefly, the ion concentration of standardized SBF solution was prepared correspond to that in the blood, which is presented in Table 1. The fluid was prepared by dissolving reagent-grade NaCl, $NaHCO_3$, KCl, $K_2HPO_4 \cdot 3H_2O$, $MgCl_2 \cdot 6H_2O$, $CaCl_2$ and Na_2SO_4 in ultrapure water, and then adjusted to pH 7.40 by using tris-(hydroxymethyl)-aminomethane [$(CH_2OH)_3CNH_2$] and HCl (Sigma-Aldrich, Saint Louis, Missouri, USA). Afterwards, BCP was immersed into SBF solution and incubated in 5%-CO_2 incubator (Thermo Scientific TM Forma Series II Water-Jacketed, Santa Ana, California, USA) at 37 °C. The solution was changed every other day. After 7 days, the treated BCPs were rinsed by PBS and sterilized for *in vitro* culture. The procedure for BSA-SBF immersion was same except the addition of BSA (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) in SBF solution. The final concentration of BSA is 70 mg/ml, which is in accordance with that in human serum (60–80 mg/ml) [11]. As control, BCP was incubated in phosphate-buffered saline (PBS, Sigma-Aldrich) 7 days.

2.2. General observation and scanning electron microscope (SEM)

BCP without any treated was measurement by a metal ruler, and BCP which was treated by PBS, SBF, BSA-SBF after 7 days could send to the Electron microscope room of Guangxi Medical University (Nanning, Guangxi, China) for scanning electron microscope.

2.3. Isolation and culture of primary osteoblasts

Primary osteoblasts were harvested from the bilateral parietal bone of 3 to 7 day' newborn Sprague-Dawley (SD) rats obtained from the Animal Resources Centre of Guangxi Medical University (Nanning, Guangxi, China) by enzymatic digestion. After rats were put to death by cervical dislocation, the bilateral parietal bones were stripped clearly with sterile gauze in a sterile environment. After the tiny connective tissue around the parietal bone was removed by 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA) (Beijing Solarbio Science and Technology Co., Ltd.), the bone was cut into pieces about 1 × 1 mm in a sterile vial and then digested with 1 mg/ml collagenase type I (Gibco BRL Co.Ltd.) in serum-free α-MEM for 3 h. After centrifugated at 1000 rpm for 5 min, isolated osteoblasts were suspended in alpha-modified Eagle's medium (α-MEM, Gibco BRL Co.Ltd., Gaithersburg, Maryland, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco BRL Co., Ltd.) and 1% (v/v) antibiotics (penicillin 100 U/ml, streptomycin 100 U/ml, Beijing Solarbio Science and Technology Co., Ltd., Beijing, China). Culture was maintained in a 5%-CO_2 incubator at 37 °C with medium changed every other day. At 80–90% confluence after about 7 days of culture, primary cells were prepared for subsequent experiments.

Table 1
The ion concentrations of simulated body fluid (SBF) and human blood plasma according to the ISO standard (10^{-3} mol/l).

Ion	SBF(pH 7.40)	Blood plasma(pH 7.2–7.4)
Na^+	142.0	142.0
K^+	5.0	5.0
Mg^{2+}	1.5	1.5
Ca^{2+}	2.5	2.5
Cl^-	147.8	103.0
HCO_3^-	4.2	27.0
HPO_4^{2-}	1.0	1.0
SO_4^{2-}	0.5	0.5

2.4. Cell proliferation assay

To investigate proliferation, number of cells was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma-Aldrich) method. Cells were digested with 0.25% trypsin/EDTA, suspend in culture medium and seeded on BCP pretreated with the three methods in the 24-well plate at a density of 5×10^4 cells/well. At day 2, 5 and 8, the assay was performed by adding 1 ml culture medium free of FBS with the final concentration of 0.5 mg/ml MTT into each well, and incubating in a 5%-CO_2 incubator at 37 °C for 4 h. After discard of the supernatant, 1 ml Dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added for formazan-crystal solubilization. After mixed thoroughly and evenly for 10 min in a dark environment, samples of 200 μl were randomly extracted from each of three parallel wells at the same experimental group and transferred to 96-well plates. The absorbance value was measured at 570 nm with a microplate reader (Thermo Scientific Multiskan GO Microplate Spectrophotometer, Helsinki, Finland). Results were shown as units of optical density value.

2.5. Cell viability assay

Cell viability of osteoblasts was determined by fluorescein diacetate [FDA; Life Technologies (AB & Invitrogen), Carlsbad, California, USA]-propidium iodide [PI; Life Technologies (AB & Invitrogen)] staining at day 2, 5 and 8 respectively. Briefly, FDA and PI stock solutions were added to cells with a final concentration of 5 μg/ml and 20 μg/ml respectively and incubated in the dark for 5 min at 37 °C. Images were captured via a laser scanning confocal microscope (Nikon A1, Tokyo, Japan).

2.6. Cell morphology observation

After being cultured for 2, 5 and 8 days, cells on BCP in all groups were permeabilized using 0.5% Triton X-100 (Beyotime Institute of Biotechnology, Shanghai, China) for 5 min, incubated in a dark environment with rhodamine phalloidin (Cytoskeleton, Inc., Denver, Colorado, USA) for 30 min, and then stained with Hoechst 33258 (Sigma-Aldrich) for 5 min. Image capturing was performed using laser scanning confocal microscope.

2.7. Alkaline phosphatase (ALP) activity assay

Cells in different groups were lysed with 500 μl RIPA Lysis Buffer (Beyotime Institute of Biotechnology), which was added together with phenylmethane sulfonyl fluoride (PMSF, Sigma-Aldrich) to give a final concentration of 1 mM before the analysis of ALP activity. Total protein concentration (mg/ml) and ALP activity (units/ml) were measured following the manufacturer's instructions with an enhanced BCA protein assay kit (Beyotime Institute of Biotechnology) and ALP reagent kit (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China), respectively. ALP levels were normalized to the total protein content. All samples were done in triplicate.

2.8. Real-time polymerase chain reaction (RT-PCR) assay

RT-PCR assay was performed to detect the expression of alkaline phosphatase (*ALPL*), bone gamma-carboxyglutamate (gla) protein (*BGLAP*), bone morphogenetic protein 2 (*BMP2*), bone sialoprotein (*BSP*), type I collagen (*COLI*) and runt-related transcription factor 2