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Influences of $LaCl_3$ on the mineral phase transformation during osteoblast mineralization *in vitro*

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ABSTRACT

Rat calvarial osteoblasts were treated with lanthanum chloride ($LaCl_3$) to explore its effect on the mineral crystalline phase during the process of osteoblast calcification *in vitro*. The results confirmed that La was readily deposited in the mineral component of the matrix. Employing high-resolution transmission electron microscopy and Fourier transform infrared microspectroscopy techniques, we demonstrated that features comparable to dicalcium phosphate dihydrate (DCPD) and octacalcium phosphate, and hydroxyapatite (HAP) were detected in the mineral phases *in vitro*. Particularly, $LaCl_3$ treatment retarded conversion from DCPD-like phase into HAP during mineralization. In addition, La was introduced in DCPD powder during wet chemical synthesis. When compared with that of La-free DCPD, the dissolution rate of La-incorporated DCPD was lower, thereby leading to a delayed DCPD-to-HAP phase transformation. Thus, it can be concluded that $LaCl_3$ treatment influences the kinetics of inorganic phase transition by decreasing the dissolution rate of DCPD.

Introduction

The biological effects of rare earth elements (REs) have drawn much attention from chemical, biological, medical, and toxicological circles (Pagano et al., 2015). REs can be taken in by humans through foodstuffs and medical agents. Although they do not cause any serious toxicosis in most parts of the world due to a low uptake into plants and a subsequent low uptake from the gastrointestinal tract (Pennick et al., 2006), situations might be quite different in some mining areas, where the contents of REs in plants (Qi and Chen, 1985), animals (Chen et al., 2000), and humans (Zhu et al., 1997) are markedly higher. Besides, RE-containing fertilizers have been used to improve the yield and quality of several kinds of crops in China since the 1970s, and RE-containing forage additives have also been on trial in aqua culture and livestock raising since the 1990s (Liu et al., 2006; Ji and Li, 2000). In addition, some RE compounds have exhibited properties that appear promising for medical uses (Lebedis et al., 2012; Fricker, 2006). Most recently, lanthanum (La) carbonate has been used for the treatment of hyperphosphatemia in renal failure and dialysis patients (Albaaj and Hutchison, 2005). Consequently, the chance has greatly increased for entrance of REs into the human body.

Lanthanum (La) has been recognized as a "bone-seeking" element owing to its similarities to Ca^{2+} in ionic radius and coordination properties (Atwood et al., 2001; Jarup, 2002). Kinetics modeling has indicated that total bone La would increase 7-fold after 10 years of orally ingested lanthanum carbonate (Bronner et al., 2008). Some metals, such as aluminum, iron, cadmium, and lead, are known to cause bone pathologies in humans and animals (D'Haese et al.,

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1999). Thus, studying the effect of La on bone health is of great interest among scientists. Accumulated La in bone mineral may either incorporate in the crystal lattice of hydroxyapatite (HAP) or simply bind to the crystal surface (Zhang et al., 2013). As bone is a metabolically active tissue undergoing continuous remodeling *via* the balance between osteoblastic bone formation and osteoclastic resorption, La may affect bone mineral by interfering with cellular activities. To assess the effect of La on bone, a low-dose, long-term experiment had been used to test the physicochemical properties of bone mineral. The findings suggested that La accumulated in bone and retarded bone maturation by reducing the crystal size, thereby reducing crystalline order and making it more prone to dissolution (Huang et al., 2006). To our knowledge, there remains a lack of data regarding the influences of La on the mineral phase; such data are essential to understanding whether La incorporation can affect bone quality and health, similar to other potential toxic trace metals.

In the present investigation, osteoblast cultures were treated with lanthanum chloride ($LaCl_3$) to monitor changes in the mineral at different stages of the mineralization process. At the selected time points, the organic matrix was removed by hydrazine, and the deposited calcium phosphate was analyzed. Based on the combined results of the high-resolution transmission electron microscopy (HRTEM) and Fourier transform infrared microspectroscopy (FT-IRM) analyses, we demonstrate that La treatment delays the formation of HAP from its precursor crystal dicalcium phosphate dihydrate (DCPD)-like phase.

1. Materials and methods

1.1. Cell culture

Osteoblasts were obtained by sequential enzyme digestion of excised calvarial bones from 2-day-old neonatal Sprague–Dawley rats (1% trypsin in PBS for 30 min; 0.2% collagenase type II in Dulbecco's Modified Eagle medium (DMEM, GIBICO, USA) for 30 min; 0.2% collagenase type II in DMEM for 90 min). The first two digests were discarded. Cells from the third digest were collected by centrifugation and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS, GIBICO, USA), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (termed complete media) at 37°C in 95% humidified air containing 5% CO_2.

All procedures were performed in accordance with the regulations laid down by the ethical guidelines of Peking University.

1.2. Mineralization assay

For the mineralization experiments, osteoblasts were plated at 50,000 cells/cm^2 in 100 mm tissue culture dishes and allowed to adhere for 24 hr at 37°C/5% CO_2 in a humidified incubator before treatment with or without 2×10^{-6} mol/L $LaCl_3$ in osteogenic media (complete media supplemented with 50 μg/mL ascorbic acid and 10 mmol/L β-beta-glycerophosphate disodium salt (GP) (Sigma-Aldrich, USA)). The cell cultures were maintained for up to 7, 14, 21 and 28 days respectively and the culture medium was replaced every 3 days.

Mineralization of the cultures was visualized directly in the culture dishes by Alizarin Red S (ARS, Sigma-Aldrich, America) staining. The stained matrix was assessed using a Nikon Diaphot inverted microscope and was photographed using a Nikon 35-mm camera (Nikon, Tokyo, Japan). Quantitative analysis of ARS staining was performed by elution with 10% (W/V) cetylpyridium chloride (Sigma-Aldrich, USA) for 1 hr at room temperature and the optical density (OD) was measured at 570 nm.

1.3. Isolation and characterization of mineral phase

Calcium phosphate crystals were isolated from the organic matrix synthesized by osteoblasts by a modified methods described by Kuhn et al. (2000). Briefly, cells and matrix layers were harvested from the culture dishes after the culture medium was removed, rinsed quickly three times with neutral pH, 0.01 mol/L Tris buffer, and lyophilized. The samples were then incubated with anhydrous hydrazine (10 mg/10 mL, Sigma-Aldrich, USA) at 4°C under rotary mixing for 24 hr and intermittent ultrasonication for about 5 min each hour to remove the organic matrix as much as possible. After 24 hr the solvent was removed and the residue was washed twice with 100% ethanol. The residue was assayed as described below.

The lanthanum (La) content of the culture was measured using inductively coupled plasma mass spectrometry (ICP-MS, D2C2; Perkin-Elmer, USA). At the indicated times, calcium phosphate crystals extracted from cell culture were subjected to 0.5 mol/L hydrochloric acid for 24 hr to solubilize the minerals, the culture was centrifuged and the supernatant was tested for La content. And the data are representative as the mean value of three parallel experiments.

HRTEM observations were carried out using a Hitachi H-9000 NAR electron microscope (Hitachi, Tokyo, Japan) with electron acceleration energy of 300 kV. The fast Fourier transforms (FFT) of the HRTEM images were created by software named Digital Micrograph (version 3.5.2; Gatan Inc., USA).

FT-IR spectra were recorded on a Nexus 470 spectrometer (Nicolet, America) equipped with a Nic-Plan TM IR microscope (Nicolet, USA). Data were collected in transmission mode, 128 scans per point at 4/cm resolution. The measurement was in the range of 4000–450/cm.

1.4. Real-time quantitative polymerase chain reaction

Osteoblasts were harvested at a density of 8×10^5 cells in circular dishes (100 mm in diameter), and then treated with or without 2×10^{-6} mol/L $LaCl_3$ in osteogenic media. After 7, 14, 21 and 28 days of seeding, extraction procedures were undertaken using a commercial Trizol reagent (Invitrogen, America) and following instructions described previously (Wang et al., 2008). Total ribonucleic Acid (RNA) samples were transcribed to complementory deoxyribonucleic acid (cDNA) using Revert Aid First Stand cDNA Synthesis Kit (ThermoFisher, America) for reverse transcription polymerase chain reaction (RT-PCR). For the quantitative RT-PCR, 40 ng of template cDNA was added to each PCR reaction. Quantitative polymerase chain reaction (q-PCR) was performed using a Stratagene MX3005P Real-time fluorescent quantitative PCR system (Agilent Technologies, America). The specific primer sequence of bone sialoprotein (BSP), osteonectin (ON), osteopontin (OPN),

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