Preparation of Functional Silicate-substituted Hydroxyapatite and Its *in vitro* Investigation of Mechanism for Promoting Bone Formation

Zhiye Qiu, Cen Chen, Jian Liu, Jianglin Wang, Shengmin Zhang*
Advanced Biomaterials and Tissue Engineering Center, Huazhong University of Science and Technology, Wuhan 430074, China E-mail: smzhang@mail.hust.edu.cn

Statement of Purpose: Silicon is an essential trace element in growing bone and is involved in initial stage of bone calcification and bone development. In recent years silicate-substituted hydroxyapatite (Si-HA) has attracted much attention and been considered as a promising bone defect repair biomaterial. Many researches demonstrated that Si-HA is able to accelerate bone defect repair and has a better effect on promoting bone mineralization than the pure hydroxyapatite [1]. However, mechanism of Si-HA's promoting bone formation remains unknown. Prolyl-4-hydroxylase (P4H) is a key enzyme in collagen synthesis and silicon could affect its activity in the physiological environment. We suppose that Si-HA may affect P4H-related pathway in collagen synthesis, thereby promoting bone mineralization. In this study, we fabricated porous Si-HA scaffolds and investigated mechanism of Si-HA for its promoting effect on bone formation.

Methods: Silicate-substituted hydroxyapatite powders with different silicate contents were prepared by wet chemical precipitation method with $Ca(NO_3)_2$ and $(NH_4)_2HPO_4$ as calcium and phosphorus sources respectively, as well as $Si(OOCH_3)_4$ as silicon source. Three Si-HA samples with different silicon content (0.4 wt%, 0.8 wt% and 1.2 wt%) were prepared. Porous scaffolds were fabricated by using paraffin spheres as pore-forming agent. To fabricate a porous Si-HA scaffold, paraffin spheres with diameter of 400-600μm were firstly added into a cylindrical polypropylene (PP) mold, and heated in a water bath at 40°C for 10 minutes to form a paraffin mold. Si-HA powders were then processed into Si-HA slurries by dispersing 10g powders and dissolving 0.1g polyvinyl acetate (PVA) in 20mL tri-distilled water with ultrasonic assistance. Si-HA slurry was filled into the space inside the paraffin mold with an additional pressure provided by a piston in the cylindrical PP mold. After that, a sintering at 1250°C for 2 hours was performed, the paraffin mold was removed along with the PP mold during the sintering, thus obtaining a porous Si-HA scaffold. Si-HA powders were characterized by X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR) and transmission electron microscopy (TEM), Si-HA scaffolds were observed by scanning electron microscopy (SEM). Bone mesenchymal stem cell (BMSCs) derived from SD rats were employed for *in vitro* evaluation of Si-HA scaffolds. Cell proliferation experiment was performed via MTT assay. Cell morphology on the Si-HA scaffold was observed by SEM as well. Mechanism investigations on bone-forming promoting effect of Si-HA were performed via immunohistochemistry staining and ELISA assay of the P4H. Leaching liquor of Si-HA was used for BMSCs culturing in the mechanism investigation. 0.1g Si-HA was immersed in 10mL DMEM and kept at 37°C for 48 hours to prepare leaching liquor. FITC-conjugated antibody for P4H (Beijing Biosynthesis Biotechnology Co., Ltd.) was used in the immunohistochemistry staining and the results were observed by fluorescent microscopy. ELISA kit for P4H (R&D Systems, Inc.) was used in the quantitative determination of the enzyme in 7 days. Pure hydroxyapatite (HA) powders and scaffolds were also prepared with the same steps described above and recruited as control group.

Results: In the XRD patterns, all the peaks correspond to the characteristic peaks of hydroxyapatite (JCPDS No. 09-0432), indicating single-phase crystals for all Si-HA and HA powders. FTIR spectra intensity of phosphate group at $472cm^{-1}$, $566cm^{-1}$ and $603cm^{-1}$ decrease along with the increase of Si content, suggest the phosphate group may gradually substitutes by the silicate group. TEM micrographs show that Si-HA samples are nano-particles with length of 150-200nm and width of 20-50nm. SEM observation of porous Si-HA scaffolds demonstrate that all the scaffolds are porous structure with pore size of 200-300μm, which is suitable for cell growth. The pore size is much smaller than the pore-forming agent, due to the shrinkage during the sintering. Pores inside the scaffolds interconnect very well and diameter of interconnect holes are 80-100μm. MTT assay results indicate BMSCs grew and proliferated well on both Si-HA scaffolds and HA scaffolds. From the SEM observation of cell morphology on the Si-HA scaffolds, it is obviously that extracellular matrix (ECM) secreted by BMSCs on Si-HA scaffold is more than the one of the control group, suggesting Si-HA may promote ECM synthesis. On immunohistochemistry staining images, green fluorescence was presented on BMSCs cultured with Si-HA leaching liquor, while no fluorescence could be observed for the control group. In the quantitative determination of P4H by ELISA, P4H increased as the increase of Si content, indicating a positive effect of Si-HA on P4H activity.

Conclusions: Si-HA powders with different Si content were prepared and porous Si-HA scaffolds with pore interconnection were fabricated. Si-HA presented good biocompatibility and bioactivity, which is beneficial for ECM synthesis of the BMSCs. Immunohistochemistry staining and ELISA assay of P4H revealed that Si-HA could increase P4H activity in the BMSCs, so as to promote collagen synthesis and finally accelerate bone-formation.

References: [1] Hing KA. Biomat. 2006;27:5014-5026.

Acknowledgements: This work was financially supported by the NSFC under the contract numbers 81071263, 30870624 and National High-Tech R&D Project of China (2006AA03Z439).