Development and Evaluation of Natural Hydroxyapatite Ceramics Produced by the Heat Treatment of Pig Bones

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Abstract

Purpose: The aim of this research was to develop and evaluate natural hydroxyapatite (HA) ceramics produced from the heat treatment of pig bones. Methods: The properties of natural HA ceramics produced from pig bones were assessed in two parts. Firstly, the raw materials were characterized. A temperature of 1,200°C was chosen as the calcination temperature. Fine bone powders (BPs) were produced via calcinations and a milling process. Sintered BPs were then characterized using field emission scanning electron microscopy (FE-SEM), X-ray diffraction (XRD), X-ray fluorescence spectroscopy (XRF), energy dispersive X-ray spectroscopy (EDX), Fourier transform infrared (FTIR) spectroscopy, and a 2-year in vitro degradability test. Secondly, an indirect cytotoxicity test was conducted on human osteoblast-like cells, MG63, treated with the BPs. Results: The average particle size of the BPs was 20 ± 5 μm. FE-SEM showed a non-uniform distribution of the particle size. The phase obtained from XRD analysis confirmed the structure of HA. Elemental analysis using XRF detected phosphorus (P) and calcium (Ca) with the Ca/P ratio of 1.6. Functional groups examined by FTIR detected phosphate (PO₄³⁻), hydroxyl (OH⁻), and carbonate (CO₃²⁻). The EDX, XRF, and FTIR analysis of BPs indicated the absence of organic compounds, which were completely removed after annealing at 1,200°C. The BPs were mostly stable in a simulated body fluid (SBF) solution for 2 years. An indirect cytotoxicity test on natural HA ceramics showed no threat to the cells. Conclusions: In conclusion, the sintering temperature of 1,200°C affected the microstructure, phase, and biological characteristics of natural HA ceramics consisting of calcium phosphate. The Ca–P-based natural ceramics are bioactive materials with good biocompatibility; our results indicate that the prepared HA ceramics have great potential for agricultural and biological applications.

Keywords: Agricultural by-products, Bioceramics, Bioresources, Natural hydroxyapatite, Pig bones

Introduction

Hydroxyapatite (HA) is a calcium/phosphate containing bioceramic and a major mineral component of bones. HA has been used as an artificial bone material due to its biocompatibility, bioactivity, osteoconductivity, nontoxicity, non-inflammatory behavior, and non-immunogenicity (De Boer 1988). However, the mechanical properties of pure HA ceramics are poor, especially in wet environments. Thus, it is challenging to make heavy-loaded implants of pure HA. Specifically, synthetic HAs were reported to be brittle with a very low fracture toughness (Oldinski et al., 2011).

In general, biological apatite has the mineral phase of calcified tissues. It is similar to synthetic HA, but with a slightly different chemical composition (Zhang et al., 2007). Many in vivo experiments with HA have confirmed the merits of using its porous structure and bioactivity for tissue ingrowth (Dong et al., 2002). HA ceramics are...
considered as one of the most promising bone substitutes because of their bone-like chemical compositions and mechanical properties (Lu et al., 1999; Tampieri et al., 2001; Sobczak et al., 2009). The crystalline phase of the sintered bone was reported to have a composition similar to that of natural ceramics. Specifically, sintered bone consisted of Ca₁₀(PO₄)₆(OH)₂ (i.e., HA) at approximately 93 wt% and β-tricalcium phosphate (Ca₃(PO₄)₂; β-TCP) at approximately 7 wt% (Yoshimine et al., 1993). Therefore, HA sourced from animal bone has a great potential to be a bone substitute (Tadic and Epple 2004).

Recently, heat treatment has been suggested for fabricating natural ceramics to obtain protein-free bone (Tas et al., 1997; Jinawath et al., 2002; Akram et al., 2014). Some researchers have attempted to synthesize HA from biological materials, including corals (Manjubala et al., 2000), eggshells (Rivera et al., 1999), cuttlefish bone (Venkatesan and Kim 2010; Kim et al., 2013), and bovine bones (Bahrololoom et al., 2009; Ooi et al., 2007; Younesi et al., 2009; Hua et al., 2010). The sintered biological apatites have an interconnected porous structure, and therefore allow for faster bone ingrowth. Moreover, the use of HA as a bone substitute is advantageous because it is non-inflammatory and causes no immunological responses (Tadic and Epple 2004).

Therefore, the objective of this study was to pinpoint the preparation and characteristics of natural HA ceramics produced from pig bones using a high heat treatment (1,200°C).

**Materials and Methods**

This study consisted of three parts: (1) sample preparation; (2) characterization of natural HA ceramics produced from pig bones at a sintering temperature of 1,200°C; and (3) a cytotoxicity test.

**Preparation of natural HA ceramics**

Pig bones were soaked in oxygenated water for 24 h to eliminate the soft tissues. Subsequently, these samples were annealed in an electric furnace (ST-01045, Daian Scientific, Korea) at 1,200°C using a heating rate of 10°C/min for 2 h. While annealing the samples in the furnace, the organic compounds of raw materials were completely removed. The sintered bones were then pulverized by a mill (A10, IKA-WERKE, Japan) into bone powders (BPs). The particles of the sintered BPs were separated using sieves (Sieve/Shaker, Daian Scientific, Korea); the mean particle size of the BPs was 20 ± 5 μm. They were then sterilized in an autoclave and stored on a clean bench under ultraviolet light.

**Characterization of natural HA ceramics**

The microstructures and particle sizes of BPs fabricated in this study were observed using a field emission scanning electron microscope (FE-SEM; SUPRA 55VP, Cal Zeiss, Germany). All samples were coated using a BAL-TEC SCD005 sputter coater for 250 s at 15 mA. The effect of the annealing temperature on the microstructural change of the samples was examined. Chemical analysis was also conducted using EDX spectroscopy at 30.0 kV. Phase analysis of the annealed samples was performed using XRD (Bruker D5005 X-ray Diffractometer, Germany) at room temperature with Cu Kα as the radiation source at a scan speed of 1 degree/min, a step scan of 10–90°C, and an angular range of 20 of 2θ (generator = 40 kV, 40 mA; λ (radiation) = 1.5406 nm). The crystalline phase compositions were identified with reference to the standard JCPDS cards available in the system’s software. The Ca/P ratios of the annealed samples were calculated from the compounds containing both elements. FTIR spectroscopy (Nicolet 6700, Thermo Scientific, USA) was performed to better understand the phase changes upon annealing and to determine HA stoichiometry deviations. For this measurement, the transmission IR spectra were recorded using KBr pellets over a range of 400–4,000 cm⁻¹ with 1 cm⁻¹ resolution.

**In vitro degradability test**

Simulated body fluid (SBF) solution was prepared according to previously reported methods (Kokubo et al., 1990). Specifically, a solution consisting of NaCl, NaHCO₃, KCl, KHPO₄·3H₂O, MgCl₂·6H₂O, and Na₂SO₄ in distilled water was mixed with HCl to arrive at a pH value of 7.4. Our specimen was soaked into 20 mL SBF solution in an incubator at 5% CO₂ and 37°C for 2 years.

**Cytotoxicity test**

Cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS, Welgene Inc, South Korea), 10 mM ascorbic acid (L-ascorbic acid), antibiotics, and sodium bicarbonate at 37°C in a humidified atmosphere with 5% CO₂ (Steri-Cycle 370 Incubator,
Thermo Fisher Scientific, USA). Human osteoblast-like cells, MG63 (KCLB 21427, Korean Cell Line Bank, Seoul National University College of Medicine, South Korea) were used in this study. A cytotoxicity test was performed with a WST-1 assay (EZ-Cytox Cell Viability Assay Kit, Daeillab Service Co., Korea). Cell morphologies were also observed by FE-SEM.

Statistical analysis
Statistical analysis was carried out using the SAS Statistical Analysis System for Windows v8.2 (SAS Institute Inc., Cary, NC, USA). Statistical significance between the control and treatment groups was compared with t-test and a Mann-Whitney Rank Sum test at \( p < 0.05 \). The data were reported as mean ± standard deviation. Each sample was measured in triplicate.

Results

Characteristics of natural HA
At an annealing temperature of 1,200°C, the colors of the sintered BPs became white (Figure 1). This color change was attributed to the calcification and resorption processes of the inorganic phase, as well as the removal of the organic matrices (e.g., protein and collagen).

![Figure 1. Color change of sintered pig bones under annealing temperature 1,200°C (a). The pig bones were then pulverized in a mill (b).](image)

![Figure 2. FE-SEM morphological images: natural HA ceramics before soaking (a, b), in vitro degradability test of BPs soaked for 12 months (c, d), in vitro degradability test of BPs soaked for 24 months (e, f).](image)
FE-SEM micrograph and EDX analysis

Figures 2(a) and 2(b) show a FE-SEM micrograph of BPs annealed at a temperature of 1,200°C. Surface morphologies of BPs after immersing into SBF solution for 12 and 24 months are shown in Figures 3(c), (d) and Figure 4(e), (f), respectively. Unlike typical features seen in the precipitates of chemically prepared bioceramics, such as HA and α-TCP, the surface morphologies revealed a porous architecture, which is created by the apparent deconstruction and removal of organic compounds (dotted regions in Figure 2 (c-f)).

Quantitative elemental EDX analysis of natural HA was conducted. As shown in Figure 3, BPs were mainly composed of calcium (Ca), phosphorus (P), oxygen (O), and minor compounds, such as aluminum (Al), magnesium (Mg).

This analysis showed that the sintered BPs have inorganic phases. The Ca/P molar ratio of the elemental composition determined by EDX was 1.603. The natural HA ceramics that consist of Ca-deficient HA have a molar
ratio less than the stoichiometric value of 1.67. The Ca/P ratio, however, was not significantly different from that of the original BPs. Nevertheless, the results may be attributable to the partial decomposition of HA to form β-TCP as confirmed by EDX analysis. The images of the EDX elemental mapping of natural HA ceramics are shown in Figure 4: Ca, P, and O (a), Ca (b), P (c), and O (d).

**XRD and XRF analysis**

The XRD peak patterns of the BPs are shown in Figure 5. This diffraction patterns show a sharp peak with the heat-treatment temperature, thus showing the extent of crystallinity of the natural HA originating from the pig bones. This pattern exhibits narrow and clear peaks similar to the pattern of the calcium phosphate oxide used as the reference (01-089-6495). It also shows the appearance of a low intensity peak at 2θ of 33.4° corresponding to calcium phosphate. The elemental composition (%) was determined by XRF (Table 2). The Ca/P molar ratio determined by a XRF analysis was 1.678, which is quite similar to the one determined by EDX. Ca and P accounted for 95% of the total mineral amount of natural HA.

### FTIR analysis

The FTIR data of BPs are shown in Figure 5. The IR spectra of BPs present the characteristic absorption peaks of HA shown in the transmittance in Figure 6. A large number of bands in the spectra (3,571–3,572 cm⁻¹, 2,072 cm⁻¹, 2,002.5 cm⁻¹, 1,480–1,500 cm⁻¹, 1,044–1,049 cm⁻¹, 1,045 cm⁻¹, and 1,023 cm⁻¹) match the bands in the HA reference spectrum. The FTIR spectra showed the presence of ions in the annealed BPs, such as phosphate (PO₄³⁻), hydroxyl (OH⁻), and carbonate (CO₃²⁻).

### Cytotoxicity assessment

Human osteoblast-like cells on natural HA ceramics were analyzed by an indirect cytotoxicity assessment.

![Figure 5. XRD pattern analysis of natural HA ceramics annealed at 1,200°C.](image)

**Table 2.** Elemental composition (%) determined by XRF

<table>
<thead>
<tr>
<th></th>
<th>Ca</th>
<th>P</th>
<th>Ca/P molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyapatite Ca₅(PO₄)₃(OH)</td>
<td>39.9</td>
<td>18.5</td>
<td>1.67</td>
</tr>
<tr>
<td>Natural HA</td>
<td>36.7</td>
<td>16.4</td>
<td>1.73</td>
</tr>
</tbody>
</table>

![Figure 6. FTIR spectra analysis of natural HA ceramics annealed at 1,200°C.](image)

![Figure 7. An indirect cytotoxicity evaluation of human osteoblast-like cells on natural HA ceramics.](image)
Figure 8. FE-SEM images of human osteoblast-like cells on natural HA ceramics at 5 days. The yellow arrow indicates the cells cultured on the natural HA substrate. The white arrows represent natural HA ceramics. Scale bar indicates 10 µm.

(Figure 7). Compared to the control, the cell viabilities was not significantly different from the experimental group ($p > 0.05$), which suggests that the natural HA ceramics posed no threat to the cells.

**Cell adhesion morphology**

Cell adhesion morphology on natural HA ceramics was observed using FE-SEM (Figure 8). The image shows that human osteoblast-like cells can be cultured with natural HA. The yellow arrow indicates the cells cultured on the natural HA substrate, and white arrows represent natural HA ceramics.

**Discussion**

Bioceramics are extensively used for the restoration and healing of bone defects. However, biocompatible bioceramics remain a challenge to clinical adoption due to the regulations regarding biocompatibility testing specified by the FDA modified ISO-10993-1 standards (Wei et al., 2008). Our study has demonstrated that BPs annealed at 1,200°C show a form similar to that of HA. The characterization of the HA phases in BPs revealed a porous architecture created by the removal of organic phases. Once annealed at 1,200°C, the colors of the sintered BPs became white. The color change was associated with the calcification and resorption processes of the inorganic phase, as well as the removal processes of any remaining protein or collagen. The chemical compositions of the sintered BPs were close to the stoichiometric Ca/P ratio of HA. XRD signatures of the BPs were similar to the stoichiometric ratios in HA’s peaks (XRD JCPDS data file No. 9-432) (Hofstetter et al., 2012). The XRD results here showed that our natural HA ceramics were not disrupted when annealed in air at a temperature of 1,200°C.

Haberko et al. (2006) investigated natural HA extracted from animal bones by heat treatment, and the Ca/P ratio turned out to be higher than the stoichiometric material. Haberko's natural HA was fabricated by heat treatment (>1,000°C) with hot NaOH solution. It was reported that the surface area of the BPs at heat treatments below 1,000°C decreased; consequently, compacts of such powders started to shrink. In this study, natural HA ceramics produced from pig bones were fabricated with a much higher temperature. The EDX, XRF, and FTIR analysis of the BPs indicate the absence of organic compounds. The BPs were approximately stable in SBF solution for two years. Moreover, an indirect cytotoxicity test on natural HA ceramics showed no toxic effects to cells. The selected temperature used for heat treatment of animal bones is of great importance to avoid disrupting the HA phase stability (Ooi et al., 2007; Zhou et al., 1993).

Kim and Cao (2002) reported that annealing temperature ranging from 1,400–1,600°C could result in the partial decomposition of the HA phase to form a minor Ca-P phase. Further heating of the calcium-deficient form of the HA phase results in the decomposition to calcium oxide phosphate (COP) and β-TCP (Bohner 2000). The FTIR spectrum analysis shows the PO$_4^{3-}$ bands of HA located at 560 cm$^{-1}$, 962 cm$^{-1}$, and 1,950–2,200 cm$^{-1}$. Moreover, FTIR spectra exhibited pronounced peaks at 630 cm$^{-1}$ and 3,570 cm$^{-1}$ due to the presence of hydroxyl groups (Ooi et al., 2007; Silva et al., 2005). As the temperature rose to 1,200°C, the OH band at 3,570 cm$^{-1}$ gradually increased in intensity (Silva et al., 2005). Thus, all organic substances could be eliminated by annealing at this high temperature.

Sintering is the key step in processing a majority of ceramic materials, and conventionally consists of two separate stages: compressing the initial powder and heating the compacted powder up to temperatures just below their melting points (Ruhé et al., 2005). Thus, atoms and molecules are set in rapid motion, and the particles coalesce in this process (Ruhhiet al., 2005). Fusion of the crystals reduces the porosity and increases
the strength and density of the final bioceramics (Dorozhkin 2010). Currently, several sintering techniques are available, such as hot pressing (Mayo 1996), microwave sintering (Katz 1992) and pressure-less and plasma sintering (Chaim et al., 2008; Lu 2008; Hensley et al., 1993). Chemical composition and variations in pressure and temperature influence the structure and composition of the sintered BPs (Ruhé et al., 2005).

The Ca–P-based bioceramics are bioactive materials with good biocompatibility and osteoconductivity. These natural bioceramics derived from pig bones potentially have the greatest advantage for clinical applications due to their stability. The value of Ca/P ratio obtained in the present work was in good agreement with other reports of HA ceramics made from pig bones.

Conclusions

Our research underpins the hypothesis that natural HA ceramics produced from annealing pig bones can be used as an effective natural biomaterial. The natural HA ceramics were prepared via the heat treatment of pig bones using a sintering temperature of 1,200°C, and their properties were investigated. In conclusion, the sintering temperature of 1,200°C positively affected the microstructure, phase, and biological characteristics these natural HA ceramics. The biocompatibility test was also evaluated using an in vitro technique; our results indicate that the prepared HA ceramics have great potential for agricultural and biological applications.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Lim et al. Development and Evaluation of Natural Hydroxyapatite Ceramics Produced by the Heat Treatment of Pig Bones

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