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Design and construction of a microporous $\text{CO}_3^{2-}$-containing HA/β-TCP biphasic ceramic as a novel bone graft material

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Abstract

Biphasic calcium phosphate (BCP) ceramics, viewed as the first-line therapeutic materials in clinical practice, are still confronted with challenges such as undesirable biocompatibility and bioactivity given their structural and chemical deficiency. In this study, we designed a novel microporous $\text{CO}_3^{2-}$-containing BCP ceramic from Salmo salar bone in a CO2 atmosphere via two-step high-temperature sintering, and its physicochemical properties and biocompatibility were explored. A without $\text{CO}_3^{2-}$- BCP bioceramic material prepared in a single gas atmosphere was used as a comparison. The two scaffolds were characterized by x-ray diffraction (XRD), fourier transform infrared spectroscopy, and scanning electron microscopy (SEM), measured the size of its porosity and specific surface area by an instrument and in vitro cell experiment was used to test its biocompatibility. Such synthetic strategy endowed the BCP products with increased porosity and specificity. Especially, the novel $\text{CO}_3^{2-}$-containing BCP exhibits a lower degree of crystallinity than that without $\text{CO}_3^{2-}$. In addition, the natural microporosity remarkably increased from 49.6% for BCP without $\text{CO}_3^{2-}$ to 64.06% for $\text{CO}_3^{2-}$-containing BCP, and the specific surface area increased from 5.829 m$^2$g$^{-1}$ to 17.161 m$^2$g$^{-1}$. Besides, two materials were non-toxic to human bone marrow mesenchymal stem cells (hBMSCs), and the $\text{CO}_3^{2-}$-containing BCP had more factor expression in the early stage of osteogenesis induction, such as Runx2 and Smad 4. Overall, microporous $\text{CO}_3^{2-}$-containing BCP were prepared via a simple preparation method from natural bone, combining the structural and chemical preponderance, as well as in vitro performance it should be considered as a promising biomaterial for further explorations in the fields of bone graft materials from natural bone to satisfy versatile clinical requirements.

1. Introduction

As a part of the search for bone graft materials with good biocompatibility, bioactivity and biosafety, biphasic calcium phosphate (BCP) ceramics consisting of hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP) have been found in a wide range of applications in tissue engineering scaffolds, implant surface coatings, bone cements, drug delivery vehicles, etc and played an important role in bone defect repair and reconstruction, orthopedics, dentistry, and craniofacial surgery [1–3]. The chemical composition and physical structure of BCP ceramics are the main factors inducing osteogenesis. The chemical composition affects the degradation and redeposition rate of ceramics. The physical structure including pore structure and surface micromorphology,
mainly affects the vascular ingrowth, calcium and phosphorus ion enrichment, local fluid motion, protein adsorption, and the forming of the attached cell.

Currently, BCP scaffold are mostly prepared by BCP powder through the holemaking process, BCP powder is usually synthesized by chemical precipitation at a temperature of greater than 1000 °C [4, 5]. The conventional method is complex, expensive and time consuming.

The chemical and physical structure of the scaffold was affected impurities may be generated in each preparation step. In view of the structure and composition of natural bone is similar to human bone, researchers have attempted to collect BCP from natural bones. Recently some studies have shown that deep-sea preparation step. In view of the structure and composition of natural bone is similar to human bone, researchers have shown that the substitution of CO$_3^{2-}$, which is most abundant among the trace elements in human bone, into HA lattices could result in lattice distortion, inhibiting the growth of large grains, decreasing the equivalent particle size, and increasing the porosity and the specific surface area [8–10]. However, CO$_3^{2-}$ is almost completely decomposed when BCP ceramics collected from fish bones were calcined at high temperature, and the as-prepared product has high crystallinity [11]. In terms of the biocompatibility and biosafety of the CO$_3^{2-}$-containing ceramics, studies have revealed that human bone mesenchymal stem cells (hBMSCs) more metabolically active when in contact with CO$_3^{2-}$. Concomitantly, the messenger ribonucleic acid (mRNA) expression of osteoblast gene markers, such as alkaline phosphatase (ALP), were upregulated under the CO$_3^{2-}$ background, indicating that CO$_3^{2-}$ promotes osteoblast differentiation and chondrocyte differentiation of hBMSCs [12]. Interestingly, CO$_3^{2-}$-containing BCP ceramics which were endowed with abundant microporosity and high specific surface area could stimulate the bone differentiation of undifferentiated stem cells by upregulating proteins, particularly those associated with osteogenesis induction. Such characteristics implied the as-described abundant-microporosity framework with high surface area may have a prominent impact on osteogenesis induction [13]. As a result, we speculate that the CO$_3^{2-}$-containing BCP(HA/β-TCP) collected from fish bone, which is endowed with abundant microporosity and high surface area, would gain great potential in preferable biocompatibility and biological function as a potential bone graft material.

2. Materials and methods

2.1. The preparation of CO$_3^{2-}$-contained BCP and pure BCP from fish bone
First, Salmo salar (origin: Norseland) bones were placed in deionized water, boiled for 2 h, and washed with flowing water. Next, the Salmo salar bones were placed in a drying oven at 70 °C for 48 h. To remove the organic components from the dried Salmo salar bones, they were placed in a quartz tube furnace for calcination, the temperature was raised to 600 °C at a heating rate of 10 °C min$^{-1}$, and the bones were kept under air for 1 h.

Next, the calcined bones were divided into two groups. The bones in group A were sintered in a quartz tube furnace, the temperature was raised to 900 °C at a heating rate of 10 °C min$^{-1}$, and the bones were kept under CO$_2$ (1 l min$^{-1}$) for 1 h. The bones in group B were sintered in a quartz tube furnace, the temperature was raised to 900 °C at a heating rate of 10 °C min$^{-1}$, and the bones were kept under air for 1 h. Finally, the temperature of the materials was reduced to room temperature.

2.2. The characterization of CO$_3^{2-}$-containing BCP and pure BCP from fish bone
To measure the phase composition and crystal structure of the inorganic components in the prepared materials, slow-scan fullname (XRD) (D8 ADVANCE, Germany) was used to determine the exact peak positions, and the working conditions were as follows: Cu-Kα as the radiation source and scanning at a step of 0.03° and a rate of 4 °/min in the angular range of 10–60°. Using SGI workstation Ccrisu2 (version 4.2) software package of American MSI Corporation and DWBS9411 software to refine the powder XRD analysis data of the two samples by Rietveld refinement, the unit cell parameters of the two groups of samples were obtained and the unit cell structure integrity was compared, both samples have a two-phase structure, the theoretical density was determined using the formula $Dx = \frac{M}{N\pi}$ (where M is the molecular weight of the sample, N is Avogadro’s number, Z is the number of molecules per unit cell and V is the unit cell volume) calculate the density of each single phase of each sample, and then calculate the calculated density of the two samples according to the formula $Dx = AD_t + BD_{\alpha}$ (where $A$, $B$ are the relative contribution ratios of the existing crystalline phases) [14–17]. The molecular functional groups of the sample, especially the CO$_3^{2-}$ vibration, were analyzed by fullname (FTIR) spectroscopy (1750, Perkin-Elmer, USA). A prepared sample and anhydrous KBr were compressed into a circular piece at a ratio of 1:50 and analyzed by an Agilent Cary 670 spectrometer with a resolution of 1 cm$^{-1}$ in the range of 4000–500 cm$^{-1}$. The calcined samples were observed by scanning electron
microscopy (SEM; JSM-5900 LV, JEOL, Japan) for microstructural analysis. The CO$_2^2$ content of the samples was analyzed as the carbon content using a fullname (CHN) analyzer (Medac, UK). The pore size distribution and porosity of the materials were measured using a mercury intrusion meter (AutoPore V9600, Micromeritics, USA). The mercury intrusion meter has an operating pressure range from 0.5 to 33 000 psi, corresponding to a pressure range of 400–0.003 μm. A cumulative mercury injection volume-aperture curve was obtained using machine mapping. According to the Washburn formula \( d = \frac{2 \gamma \cos \theta}{p} \) (\( d \) : porosity, \( \gamma \) : surface tension of mercury, \( \theta \) : contact angle and \( p \) : mercury pressure; where the surface tension of mercury \( \gamma \) is generally 480 dyn cm$^{-1}$ during general testing, and the contact angle \( \theta \) between mercury and porous body is 140°) [18], the instrument can automatically calculate the pore distribution of porous materials. Using a specific surface area analyzer (TriStar II 3020, Micromeritics Company, USA), the specific surface area of the samples was assessed using a nitrogen adsorption method.

2.3. Conventional culture of hBMSCs
5–10 ml of bone marrow was taken from the posterior superior iliac spine with approval from the Health Human Research Ethics Committee of the Naval Medical University, and Ficoll lymphocyte separation solution containing approximately 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin, P/S). The cells were counted, inoculated into a culture containing approximately 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin/streptomycin, P/S). The cells were counted, inoculated into a culture flask of 25 cm$^2$ at a concentration of 1.5–2.0 × 10$^5$ cm$^{-2}$, and cultured in an incubator (KeyGEN Biotech, China) at 37 °C with 5% CO$_2$ for primary culture. After 24 h, the unattached cells were discarded, and the medium of the attached cells was changed every two days. S... was carried out when the degree of cell fusion reached 70%–80%. The cells with good growth from passages 3–5 (P3–P5) were used for cyto compatibility and osteogenic efficacy testing.

2.4. The preparation of extraction medium of two BCP materials
The method of extraction medium preparation was in accordance with International Standard ISO 10993-12:2007. First, group A and group B were separately added to DMEM containing 10% FBS and 1% antibiotics (P/S) (the ratio of material mass to extraction medium volume was 0.1 g mL$^{-1}$), then incubated in a 37 °C constant temperature oscillator for 24 h, sterilized with a 0.22 μm filter, and sealed in a sterile bottle. Extraction media of groups A and B were prepared and were finally placed in a 4 °C refrigerator for later use.

2.5. Cytotoxicity assessment
The cytotoxicity of the two BCP materials was evaluated using a Cell Counting Kit-8 (CCK-8; GLPBIO, Montclair, USA). In 96-well plates, 3000 hBMSCs were added to each well for 24 h of preculture. The experimental group was supplemented with 100 μl of extraction medium of group A; the control group, with 100 μl of extraction medium of group B; and the blank control group, with 100 μl of DMEM containing 10% FBS and 1% P/S. Then, the cells were cultured in an incubator for 1 day, 2 days, 3 days or 4 days. Before the test, 10 μl of CCK-8 solution was added to each well and cultured in the incubator at 37 °C under 5% CO$_2$ for 4 h. The absorbance at 450 nm (OD value) was thereafter measured.

2.6. Western blotting analysis
In 6-well plates, 3000 hBMSCs of P3 were added to each well and cultivated in 2.5 ml of culture medium (DMEM with 10% FBS and 1% P/S) in an incubator for 24 h at 37 °C under 5% CO$_2$. Group A was supplemented with 500 μl of extraction medium of group A, and group B was supplemented with 500 μl of extraction medium of group B. After 7 days of incubation with the extraction media of the two BCP materials, the hBMSC extracts were lysed with fullname (RIPA) buffer (P0013B, Beyotime, China), separated from the same amount of protein sample by 12% SDS-PAGE electrophoresis and transferred to a hybridization nitrocellulose filter (Merck Millipore, Germany). The membranes were incubated with primary antibodies (anti-RUNX2 and Smad 4) overnight at 4 °C. The next day, the membranes were incubated with secondary antibodies (HRP-conjugated goat anti-rabbit IgG (H + L) (Thermo Scientific, USA)) at room temperature for 2 h. On an x-ray film, signals were developed with electrochemiluminescence (ECL; Beyotime, China), and protein bands were visualized.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein was used as a reference.

2.7. Osteoinductive differentiation of hBMSCs
In 6-well plates, 3000 hMSCs of P3 were added to each well and cultivated in 2.5 ml of culture medium (DMEM with 10% FBS and 1% P/S) in an incubator for 24 h at 37 °C under 5% CO$_2$. The cells reached 80% fusion, and the growth culture medium was removed. Then, 3 ml of osteogenic induction culture medium containing 50 mg l$^{-1}$ ascorbic acid, 10 mmol l$^{-1}$ β-sodium olate, 10–8 mol l$^{-1}$ dexamethasone and 500 μl of extraction
medium of group A or 500 μl of extraction medium of group B was added. When osteogenesis was induced and the cells had been cultured for 21 days, they were washed with PBS, stained with alizarin red for 3–5 min, washed 2–3 times with PBS, and observed under an inverted microscope.

2.8. Statistical analysis
The values reported were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using Student’s t-test or one-way analysis of variance. Statistical differences are presented as (*) for p < 0.05, (**) for p < 0.01, (***) for p < 0.001, and (****) for p < 0.0001.

3. Results and discussion
3.1. The preparation and characterization of CO₃²⁻-containing BCP and pure BCP
The characterization of the as-synthesized CO₃²⁻-containing BCP and pure BCP was determined in the first place. XRD patterns of two categories of BCP ceramics (figure 1) suggested that the main crystalline phase is HA, whereas the HA peak of group A is offset from the HA peak on the standard card. Moreover, the full width at half maximum of group A was significantly larger than that of group B, indicating that the degree of crystallization of group A is lower than that of group B. As speculated, the difference is mainly due to the lattice distortion caused by the substitution of CO₃²⁻. In addition to the fact that the unit cell volume of CO₃²⁻ is larger than that of PO₄³⁻ could be distinctly observed in morphology and size, the formation of a B-type substitution when substitution occurs. This distortion could suppress the further crystallization of HA, which downgrades the degree of crystallization [8–10].

The lattice parameters and theoretical density parameters obtained by Rietveld structure refinement were listed in table 1. Due to the amount of CO₃²⁻ incorporation, the a-axes of the lattice parameters have decreased significantly from 9.4845 nm to 9.2942 nm, which showed that the incorporation of CO₃²⁻ affects the crystal structure of HA, and the lattice structure tends to be incomplete. The reduction in theoretical density calculated by x-ray diffraction means that the arrangement of atoms in the unit cell tends to be loose and irregular, and the interaction between the atoms is weakened, the reduction in density indicates less stability. This is consistent with the result that the x-ray diffraction peaks broadened and shifted [14–17].

Table 1. Variation of lattice parameters and theoretical density in Group A and B.

| Group | Lattice parameters (nm) | Theoretical density (g/cm³) |
|-------|------------------------|----------------------------|
|       | a          | b          | c          |                       |
| A     | 9.2942     | 9.2942     | 6.9294     | 2.882                 |
| B     | 9.4845     | 9.4845     | 6.8752     | 3.114                 |

Figure 1. X-ray diffraction spectrum of the group A, B; In the figure, the Δ refers to the characteristic diffraction peak of HA, the ○ refers to the characteristic diffraction peak of β-TCP.
Quantitative experiments were further conducted for structural analysis. As shown in Table 2, the concentration of β-TCP in group A was higher than that in group B. The reason accounting for this phenomenon may be that the decomposition temperature of HA was affected by the presence of CO$_2$O$_3$.$^2$ The stoichiometric amount of HA will decompose above 1100 °C into β-TCP, but the decomposition temperature of carbonated hydroxyapatite (CHA), approximately 700 °C, is apparently lower than this temperature. Consequently, it could be concluded that the β-TCP content in group A may be partially derived from CHA.$^{19,20}$

In order to confirm the existence of CO$_3$.$^2$ in our prepared samples, FTIR spectra of groups A and B and unsintered fish bone were tested (Figure 2). The characteristic peak of β-TCP appeared at 1122 cm$^{-1}$ and 959 cm$^{-1}$.$^{19,20}$ Specifically, the IR spectrum of group A exhibited high-intensity bands at 1465 cm$^{-1}$ and 875 cm$^{-1}$, which correspond to characteristic peaks of CO$_3$.$^2$.$^{21}$ By contrast, there were almost no CO$_3$.$^2$ bands in the spectrum of group B. It could be explained that during the heat treatment at 823-1000 °C under an atmosphere of CO$_2$ gas, CO$_3$.$^2$ entered the HA crystal and replaced some of PO$_4$.$^3$ to form a B-type substitution. The weak characteristic peaks of the amide I and II bands could be observed at approximately 1654 cm$^{-1}$ and 1544 cm$^{-1}$, in groups A and B, respectively.$^{22}$ Previous studies have illustrated that the width and sharpness of a characteristic absorption band in an IR spectrum can reflect the degree of crystallinity of a mineral. When the degree of crystallization is low, the internal structure of the mineral becomes irregular, the symmetry is reduced, and some broad and blunt peaks are formed.$^{23,24}$ As the degree of crystallization increases, the original wide frequency range of the absorption band is reduced, and the absorption band is sharpened. The comparison of the FTIR spectra between group A and B signified that the peak of PO$_4$.$^3$ at 1100-900 cm$^{-1}$ of group B had a relatively sharper shape, while in the spectrum of group A, the peak had a wider shape. This result is consistent with the XRD data shown in Figure 1.

Table 2. Quantitative analysis of phase composition of materials in groups A and B (HA/β-TCP, %).

| Group | HA   | β-TCP |
|-------|------|-------|
| A     | 54.7 | 45.3  |
| B     | 68.82| 37.18 |

Next, the CO$_3$.$^2$ content was further determined by a CHN analyzer.$^{25}$ It turned out to be 1.91wt% in group A and 0.068wt% in group B, respectively. Combining both qualitative and quantitative analyses, CO$_3$.$^2$ content in the sample A was proved to be close to that in bone tissue.$^{26}$

The morphological characteristics of the samples were then confirmed. It could be observed from SEM images (Figure 3) that both BCP products from fish bone (group A and B) have interconnected natural
macroporous structures ranging from 0.1 to 3 mm. Furthermore, the particle size in group B are significantly larger than that in group A (figure 4), suggesting that the incorporation of CO$_3^{2-}$ into the HA lattice changed the structure thus inducing the decrease of crystallinity. The structural features in SEM images coincide with the crystallinity results observed from the XRD patterns.

The pore size distribution and porosity of the products in groups A and B were estimated by a mercury intrusion meter. Figure 5(a) shows the cumulative mercury injection volume-aperture curve obtained using machine mapping. Figure 5(b) shows that the products from both groups have a pore size range between 0.15 and 0.03 μm and that product in group A has a sharper peak shape than group B, indicating that there are higher densities of micropores in group A. In addition, the porosity of the products in group A and B was calculated to be 64.06% and 49.6%.

Figure 3. Submicron pore structure of Group A and B under 120x Scanning electron microscope.

Figure 4. The crystallinity of group A is less than that of group B under 30.0kx Scanning electron microscope.

Figure 5. (a): Cumulative mercury injection curve; (b): Pore size map; the group A has more microspores than group B.
And the specific surface area was 17.161 m$^2$ g$^{-1}$ and 5.829 m$^2$ g$^{-1}$, respectively, as measured by BET analysis using the nitrogen adsorption method. This finding is in accordance with the results obtained by SEM and the Mercury intrusion meter.

According to the results of SEM images, porosity and specific surface area calculation, we can conclude that the addition of $\text{CO}_3^{2-}$ into BCP changes its physical structure so that it was endowed with a lower degree of crystallinity, more microspores and larger specific surface area. The degradation rate of BCP with a lower degree of crystallinity would be more biologically compatible than that of BCP with a higher degree of crystallinity. The macroporous structure gains the morphological and mechanical advantages in supporting cell and blood vessel ingrowth. Meanwhile, local osteogenic induction could be provided with preferable structural basis, given that micropores could allow body fluid penetrating into the implant to enhance its biological activity. Notably, its surface micropores can also function as a regulatory material for degradation, redeposition and promotion of material osteoinductive key factors of conductivity [27–30]. Studies have shown that compared to micropores alone, the materials with microporous and macroporous have superior osteoinductivity [31]. Besides, specific surface area has a significant promotion on cell adhesion and proliferation, as well as osteogenic differentiation. The larger the specific surface area of the material is, the greater the amount of protein it can adsorb [32–35]. As a consequence, the as-described properties of $\text{CO}_3^{2-}$-containing BCP is expected to be a desirable candidate for biomedical application.

3.2. Cytotoxicity test
Given the excellent properties $\text{CO}_3^{2-}$-containing BCP, we further evaluated its performance in cells. Initially, the cytotoxicity of the two BCP materials was assessed by CCK-8 test. As shown in figure 6(a), with prolonging culture time, the OD values in each group increased, indicating that the hBMSCs could proliferate under the material background. On the first day of co-incubation, although the OD values in the two groups were higher than those in the blank control group, the difference was not statistically significant. On the second day, the OD values in the two groups were significantly higher than that in the blank control group, suggesting that the extracts of these materials could promote cell proliferation. Specifically, the promotion effect of group A was more obvious than that of group B. This finding indicated that hBMSCs were more metabolically active when in contact with CO$_3^{2-}$, which was also validated in other researches [36, 37].
3.3. Effect on osteoinductive differentiation of hBMSCs

To understand the molecular mechanism of its proliferation effect, the expression of genes involved in osteogenesis was tested. It has been well demonstrated that osteogenic differentiation can cause the expression of several regulated genes and specific marker genes at different stages. Runx2, an early osteogenic differentiation marker, can activate osteoblast differentiation from mesenchymal precursor cells [38]. Figure 6(b) shows that after 7 days, compared to that in group B, the expression of Runx2 in group A was significantly enhanced, validating that CO$_3^{2−}$ promotes osteogenic differentiation of hBMSCs. This finding is consistent with the results of a recent study, which proved that after 21 days of culture, hBMSCs cultured in medium containing CO$_3^{2−}$-substituted HA could produce more total protein than those cultured in a medium containing HA without CO$_3^{2−}$ [12].

To investigate the mechanisms that how CO$_3^{2−}$ promotes osteogenic differentiation, we detected the expression of Smad4 [39]. It has been found that CO$_3^{2−}$ functions as a chemical stimulus of hBMSCs by activating the MAPK signaling pathway and thus induces osteogenic differentiation of these cells. There are two types of pathways that promote osteogenesis: Smad-dependent pathways and Smad-independent pathways [40]. Studies have confirmed that the main difference between the two mechanisms is Smad4. As shown in figure 6(b), after 7 days, the expression of Smad4 was significantly enhanced in group A compared to group B. As a result, CO$_3^{2−}$ was considered to promote osteogenic differentiation in hBMSCs mainly via a Smad-dependent pathway.

To further demonstrate that CO$_3^{2−}$ promotes osteogenic differentiation of hBMSCs, we performed osteogenic induction of hBMSCs for 21 days and stained them with alizarin red. As shown in figure 6(c), group A has more red calcified junctions than did group B. Combining the results of cytotoxicity tests and osteogenic activity assays, it could be elucidated that the as-synthesized CO$_3^{2−}$-containing BCP can promote the metabolic activation and osteogenic differentiation of hBMSCs.

4. Conclusion

In conclusion, we designed a microporous CO$_3^{2−}$-containing BCP from Salmo salar bone via two-step sintering by a high temperature calcination method under different gas atmospheres, the CO$_3^{2−}$ content calculated to be 1.98 wt%, which is close to that in human bone tissue (3 ~ 8 wt %). The as-prepared biomaterial was endowed with a lower degree of crystallinity than that without CO$_3^{2−}$, which adjusts the rate of new bone formation to match the rate of material degradation. Especially, its unique macroporous structure gains the morphological and mechanical advantages in supporting cell and blood vessel ingrowth; the natural microporosity remarkably increased from 49.6% for BCP without CO$_3^{2−}$ to 64.06% for CO$_3^{2−}$-containing BCP, microporosity is the key structure for osteogenesis induction, and the specific surface area increased from 5.829 m$^2$g$^{-1}$ to 17.161 m$^2$g$^{-1}$, the larger specific surface area has more significant promotion on cell adhesion and proliferation, as well as osteogenic differentiation. Besides, in vitro experiments the CO$_3^{2−}$-containing BCP had more expression of Runx2 and Smad 4 confirmed that the CO$_3^{2−}$-containing BCP can improve the metabolic activity and osteogenic differentiation of hBMSCs. Such inherent characteristics and in vitro performance of CO$_3^{2−}$-containing BCP make it a desirable biomaterial for further explorations in the fields of bone graft materials to satisfy versatile bioapplication requirements.

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