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Comparison of Purity and Properties of Hydroxyl Carbonate Apatite Extracted from Natural Thigh Bone by Different Physiochemical Methods

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ABSTRACT

New approaches to extracting natural hydroxyl carbonate apatite from bio waste of bovine bones cortical femur have been developed. To extract pure and natural bio ceramics, three different treatments have been applied: (1)-Calcination heat treatment at temperature of 700 °C, (2)-Alkaline hydrothermal at temperature of 275°C and (3)- Pressurized low polarity water hydrothermal at temperature of 250 °C. Raw bovine bone and obtained apatite have been characterized by Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction, differential thermal analysis (DTA), thermogravimetric analysis (TGA), fluorescent microscopy and scanning electron microscopy (SEM). Under heat treatment process, mainly all the organic component such as collagen was removed at temperature of 600 °C and a hydroxyl carbonate apatite was obtained. The presence of carbonate groups led to increasing the biocompatibility and will be preferable for orthopedic and medical usages. The degree of crystallization of powders is increased by increasing of temperature from 250°C for pressurized low polarity water processing to 600°C for calcinations treatment. Growth behavior and adhesion of cultured umbilical cord mesenchymal stem cells on the surface of 2-D hydroxyapatite scaffolds derived by these three different methods have been investigated. The results show that the stem cells could survive and attach on surface of derived carbonated hydroxyapatite scaffolds by calcinations.

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1. INTRODUCTION

Hydroxyapatite (HAp) and calcium phosphates are the major constituents of body's bone and is extensively used as a bioceramic for bone replacement, repair of the skeletal system, joint, orthopedic usage, teeth and other medical application [1-5] due to good biocompatibility, bioactivity behavior and non-immunogenicity properties [6-10]. HAp with the chemical formula of $Ca_{10}(PO_4)_6(OH)_2$ has a theoretical composition of 39.68 wt.% Ca, 18.45 wt.% P; and Ca/P molar ratio of 1.667. Natural apatite are non-stoichiometric (Ca/P ratio over 1.67) and contain carbonate groups (CO_3) and usually partial bioactive ions (e.g. Mg, Na, K, SiO_2, etc.) which localized interstitially and substitutionary into its structure, respectively. Carbonate groups are the most abundant substitution in bone minerals and might be found in two types of A (substituted in position of OH^-) and B (substituted in position of PO_4^{3-}) replacement. The type of B is the most frequent substitution in natural bones [11]. However the amount of these ions is very low, but plays a vital role in the biological reactions associated with bone metabolism [12] during the life of bone growth. Generally, the amount of hydroxyapatite and organic compounds in bones are between 65-70% and 30-35%, respectively. One of the most important organic compounds in bones is collagen. Collagen and its subgroups constitute over 95% of organic compounds in bones, and the other content exist in small concentrations (e.g. keratin sulfate, lipids, phospholipids, triglycerides, fatty acids, cholesterol,

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chondroitin sulfate, etc.). One strategy to improve non-autograft materials and maintaining their advantages is to processing them from natural sources (e.g. fish bone, bovine bone, teeth, etc.) [13-20] by traditional calcination heat treatment [21-28], novel techniques of alkaline hydrothermal, pressurized low polarity water and other methods [29-32]. In comparison with allogeneic bone, obtained hydroxyl carbonate apatite from xenogeneic bone (generally bovine bones) have advantage of lower cost, more available, easier preparation and environmentally preferable [10, 16, 17, 24]. We have synthesized the hydroxyapatite by hydrothermal method in addition of calcination treatment in order to decomposition and hydrolysis of organic compound. It has been reported that the suitable temperature for combustion and completely removing the organic components from bovine bones is between 500 -650 °C [25-27]. It is generally confirmed that heat treatment at elevated temperatures (higher than 900°C) lead to formation of new mineral phases such as, CaO, $(CaO)_3PO_4$, in structure of apatite [22, 23, 28]. Therefore, temperature of heat treatment has an important effect on properties of obtained Hap. The present works focus on preparation and characterization of HAp powder obtained by three various methods: (1) alkaline hydrothermal treatment, (2) pressurized low polarity water hydrothermal treatment and (3) calcination heat treatment from bovine bone. The objective of our study is reducing of synthesis temperature by using a new approaches to extracting natural hydroxyl carbonate apatite from bio waste and replacement of OH^- and PO_4^{-2} by carbonate groups in derived carbonated hydroxyapatite scaffolds (HCAp) in order to increase the bioactivity of bio-ceramic.

2. MATRIALS AND METHODS

2. 1. Raw Material Preparation The fresh thighs of adult bovine bone (2 years – male) were used as raw materials. The thigh is broken and the inside fats, impurities and spongy parts were removed from compact part. The samples of $1\times1\times1$ cm^3 were boiled in distilled water for 2-3h and were immersed in acetone solution with temperature of 4-6 °C during 36h. Finally, the samples have been dried at 150 °C for 48h in oven and grinded by fast milling to produce the particles with sizes less than 500 µm (Figure 1).

2. 2. Synthesis of Hap In calcination processing (Named: Thermal), powdered raw material was heated at temperature of 700°C during 6 hours under atmospheric condition.

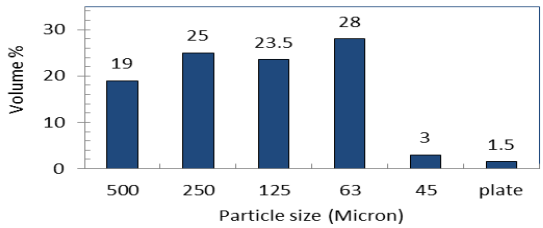

Figure 1. Distribution of particle size for fast milled bovine bone.

In alkaline hydrothermal method (Named: NaOH), raw bovine bone powders were suspended in 45 wt. % NaOH solution with solid/liquid ratio of 1:30 and heated at 275 °C for 4 hours in a cylindrical autoclave (110 mm in height and 90 mm in diameter). The yellowish water, formed by decomposition and hydrolysis of collagen and organic components, and remained powders were filtered and dried at 100°C for 2 hours. In aqueous hydrothermal process (Named: PLPW), raw powder of bovine bone were added to pressurized low polarity deionized water with solid/liquid ratio of 1:50 and heated at 250 °C for 3 hours in the same autoclave.

2. 3. Culturing of Stem Cells on 2-D Scaffolds The pellets with dimensions of 10mm in diameter and 2mm in thickness were prepared from powders obtained by three different methods by using PVA binder and cold isostatic pressing (CIP) technique with pressure of 30bar. The pellets were aged for 36 hours at temperature of 200°C. Stem cells were cultured on each scaffold in the presence of dulbecco′s modified eagle medium (DMEM) containing 10% Fetal Calf Serum (FCS), 1% L-glutamine and 1% penicillin/Streptomycin for 24 h. To evaluate quality and quantity of cultured cells on surface of scaffolds (Structure, the viability and growth of cultured stem cells), the cultured samples were fixed with 4% paraformaldehyde and they were washed with phosphate buffer saline (PBS) and immersed in 10ng/mL DAPI[1] ($C_{16}H_{15}N_5$ – Sigma Aldrich) for 5 min. Finally, the scaffolds were washed and observed under a fluorescence microscopy (Nikon-Eclipse E600W).

2. 4. Powders Charactrizations Fourier-transform infrared spectroscopy (FTIR Bruker Tensor 27, ATR, Germany) was used to investigate the functional groups such as hydroxyl groups (OH^-), carbonate (CO_3^{2-}), anion phosphate (PO_4^{3-}) and organic compounds of powder samples (raw and treated bones). The spectrum was recorded between 400 and 4000 cm^{-1}. For this measurement, the absorbance IR spectra were recorded using KBr (Merck) pellets (3 mg sample and

[1] 4',6-diamidino-2-phenylindole

300 mg KBr). X-ray diffraction (XRD) was used to characterize the chemical composition, and structure of the materials before and after treatments at room temperature, (Philips X'Pert-MPD, CuK_{α}, λ=0.15405 nm, 20mA, 40kV). Scans speed were performed with 0.05°/sec in range of 2θ values from 20° to 60°. The phase composition of the powders was monitored by comparing acquired spectrums with peaks identified in the joint committee on powder diffraction standards (JCPDS) cards available in the system software [33]. Thermogravimetric analysis (TGA) and differential thermal analysis (DTA) (STA 504, Germany) applied to study the thermal reactions and stability of bovine bone at range of 25 °C to 1000 °C with heating rate of 10 °C/min under ambient oxidizing atmosphere. Scanning electron microscopy (SEM– VEGA\\TESCAN–LMU) was used to observe the morphology, distribution of particles size of obtained HAp powders.

3. RESULTS AND DISCUSSIONS

3. 1. FTIR Analysis The bovine bone is a composite with extracellular matrix of calcium phosphates nano-crystals and fibers of organic of collagen [18]. FTIR spectroscopy is one of the best analysis methods for structural investigations of organic and inorganic compounds. FTIR spectrums of raw bovine bone and treated bones that are shown in Figure 2, have detected the amorphous and different crystalline phase (Functional group of carbonate, phosphate and hydroxide).

Figure 3a shows the absorption bands of collagen vibration namely at 1222 cm^{-1} to 1394 cm^{-1} for raw bon powder. These absorption bands have been disappeared for the treated samples indicating that all organic compounds have been removed from bovine bone powders (Figure 3b.). For all of the bands of hydroxyl (O-H), phosphate and apatite, the intensity is increased by increasing of processing temperature, while, the intensity of O-H bands related to adherent water (1647cm^{-1} and 3450cm^{-1}) decreased simultaneously. As expected, hydroxyl groups were identified in wavelength of 3570 cm^{-1} and 638 cm^{-1}. It should be noticed, the low band observed in spectra at 638 cm^{-1} for synthesized Hap could not be identified in raw bovine bone sample.

Additionally, the typical bands of substituted carbonates have localized in sloping and vertical faces of the tetrahedron sites of phosphate (type B) in the apatite lattice. In effect, both O-H in the *c*-axis channel of apatite (type A carbonate) and phosphate group (type B carbonate) can be substituted by carbonate ions [11]. Type B carbonate is characterized by a single band at 871cm^{-1} (ν_2, CO_3) and double band at 1410 cm^{-1} and 1455 cm^{-1} associated to type A carbonate (ν_3, CO_3) (Table 1). Phosphate vibrations are detected in range of 1016 cm^{-1} to 1126 cm^{-1} and HAp vibration detected at 476, 576, 607 and 964 cm^{-1} (Figure 2).

3. 2. XRD Analysis The XRD patterns of bovine bone and treated bone powders are shown in Figure 4. The crystallization degree of raw bovine bone is too low that can be related to presence of collagen compounds in matrix of bone and amorphous structure of bone.

By increasing of temperature from 250°C for PLPW, to 700°C for thermal processes, the crystallization degree of powders began to increase alternatively. According to Pang equation [26], the crystallization degree of crystalline phases was calculated (Table 2):

$$X_c = 1-[V_{(112/300)} / I_{300}] \quad (1)$$

where, I_{300} is the intensity of (300) reflection and $V_{112/300}$ is the intensity of the hollow between (112) and (300) reflections.

In apatite lattice, due to the presence of carbonate ions interstitially in structure, the planar spacing of tetrahedron phosphates increased and broadening of main peaks take place. Total error in d-spacing for thermal process is trace but for two other samples is higher that can be related to higher presence of carbonate ions in structure of NaOH and PLPW samples (Table 3). It has been reported that, at elevated temperature of processing, the carbonate contents began to decompose in gaseous shape like CO_2 [24]. For stoichiometric hydroxyl carbonate apatite (HCAp), chemical formula is presented in form of Ca_{10} (PO_4, $CO_3OH)_6$ $(OH)_2$.

3. 3. DTA–TGA Analysis According to the results of DTA-TGA analysis (Figure 5a,b), there is a endothermic reaction related to the evaporation of incorporate water molecules at temperature of 200°C. With increasing of temperature, the significant exothermic peak related to the burning of organic compound is appeared in range of 260°C to 628°C (Figure 5a). By increasing of temperature at higher than of 600 °C, the endothermic reaction due to dissociation of inorganic compounds such as $Mg(OH)_2$, $Ca(OH)_2$, and $MgCO_3$ is take placed and a significant endothermic peak is appeared at 800 °C. This endothermic reaction is related to the interaction between carbonate (CO_3) and phosphate groups (PO_4). Due to the presence of B-type carbonate, at elevated temperatures, the following reaction takes place [24]:

$$P_2O_7^{-4} + CO_3^{-2} \rightarrow 2PO_4^{-3} + CO_2 \quad (2)$$

The DTA curve proves that the organic contents (e.g. collagen and fats) is completely removed from the matrix of bone while, the carbonate ions still present in structure of HAp and stable up to temperature of 800°C.

TABLE 1. FTIR Main carbonate spectra

HCAp	υ_2 CO_3 group		υ_3 CO_3 group			
			Major		Minor	
	Wavelength, cm^{-1}	Intensity	Wavelength, cm^{-1}	Intensity	Wavelength, cm^{-1}	Intensity
Raw bone	881	0.00099	1471	0.01	1434	0.01
PLPW	875	0.22	1423	0.56	1475	0.55
NaOH	879	0.08	1481	0.37	1425	0.35
Thermal	879	0.07	1475	0.31	1456	0.32

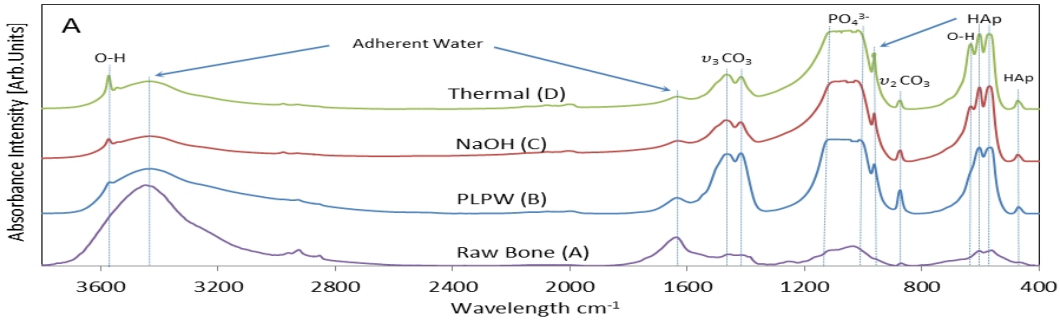

Figure 2. FTIR spectra of (a) raw bone, (b) PLPW, (c) NaOH, (d) Thermal

TABLE 2. Crytallization degree of treated bones as founction of the processing temperature.

Type of Process	Crystallization, X_C %	Temperature °C
Thermal	57 %	700
NaOH	22 %	275
PLPW	6 %	200
Raw bone	0.9 %	37

TABLE 3. Planar spacing of treated bovine bone by three different processes compared with JCPDS No.4-697

	d (nm)			
Plane(h k l)	JCPDS(4-697)	PLPW	NaOH	Thermal
(200)	0.408	0.403	0.403	0.405
(111)	0.391	0.387	0.384	0.386
(002)	0.344	0.342	0.342	0.343
(102)	0.318	0.316	0.314	0.316
(210)	0.308	0.306	0.305	0.307
(211)	0.282	0.278	0.278	0.279
(300)	0.271	0.271	0.270	0.271
(202)	0.262	0.262	0.261	0.262
(301)	0.252	0.253	0.251	0.252
(310)	0.225	0.225	0.225	0.225
(311)	0.214	0.213	0.213	0.214
(113)	0.206	0.205	0.205	0.205
(203)	0.200	0.199	0.199	0.199
(222)	0.192	0.193	0.193	0.193
(312)	0.189	0.188	0.188	0.188
(213)	0.183	0.183	0.183	0.183
(321)	0.180	0.180	0.179	0.180
(410)	0.177	0.177	0.177	0.177
(402)	0.175	0.175	0.174	0.175
(322)	0.164	0.163	0.163	0.164
	T.Error	0.0011	0.0017	0.00095

TABLE 4. Percentage of weight losses of raw bovine bone

	Weight loss (%)
Amount of evaporated waters (25-250 °C)	12.11
Amount of removed organic and inorganic contents (260-628 °C)	27.88
Amount of removed carbonate (700-1000 °C)	1.25
Total weight losses	41.24

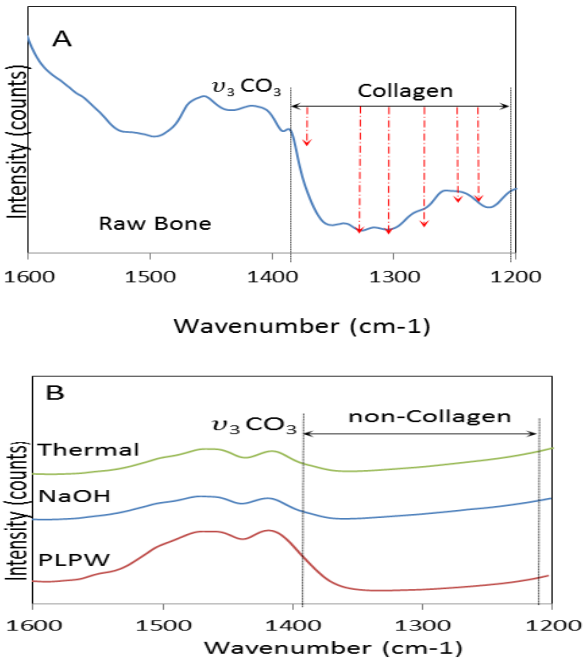

Figure 3. FTIR spectra of (a) raw bone: collagen vibration, (b) treated bone by three different processes.

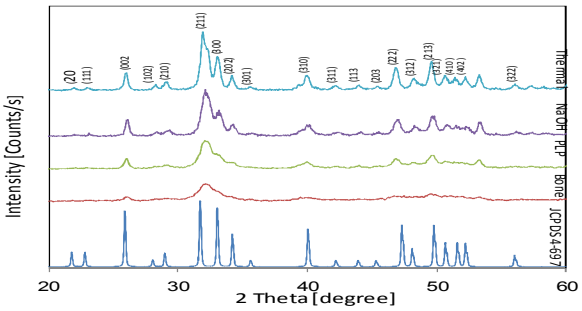

Figure 4. XRD patterns of raw bone and treated bone by three different methods

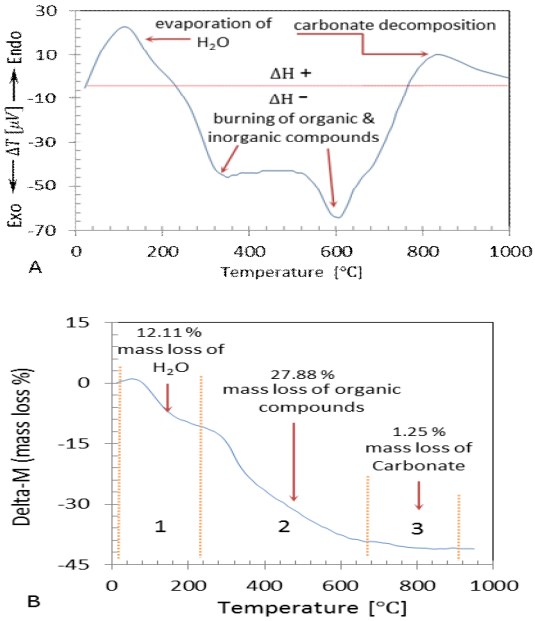

Figure 5. Thermal analysis of raw bovine bone; (a) DTA, (b) TGA

TGA curve (Figure 5b) show three stage of weight losses for raw bovine bone: (1) from 54°C to 250°C: evaporation of water, (2) from 260°C to 628°C: burning of organic and inorganic compounds, (3) higher than of 650 °C: decomposition of carbonate compounds. The percentage of weight loss of each section is shown in Table 4.

3. 4. Observation of Fluorescence and Scanning Electron Microscopy Figure 6 shows the morphology of the different synthesized powders. The morphology for all of the powder is composed of agglomerates with large distributions of size. Microstructural observation of powders by high resolution shows that the agglomerated particles are composed of fine particles in scale of nanometer (nanoparticles of ~80 nm). The morphology of the powder obtained by calcination heat treatment process is and more agglomerate and coarser than the others (Figure 6c). Observations by a fluorescence microscope of cells stained by DAPI show the cells could be attached on all scaffolds. However, the cell population shows difference in number. The number of cells cultured on the scaffold prepared by thermal process was more populated than the others (Figure 7). On the other hand, proliferation and viability of the cells is higher for the hydroxyapatite scaffolds prepared by thermal calcinations. SEM observations show that the morphology of cultured cell on surface of scaffold prepared by thermal processing is also different. These cells have a round-shape morphology (Figure 8c), The cultured cells on the scaffolds prepared by alkaline hydrothermal and aqueous hydrothermal have a expanded state and a morphology of satellite-shape (Figure 8a, b).

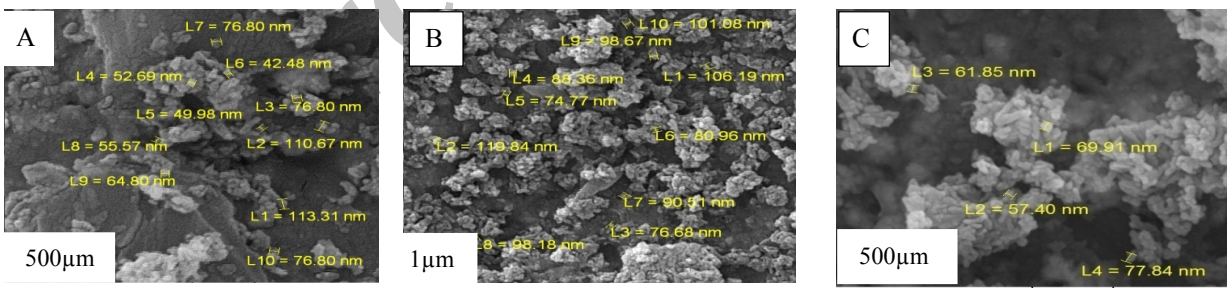

Figure 6. SEM micrographs of the obtained powders (a) PLPW (b) NaOH (c) Thermal

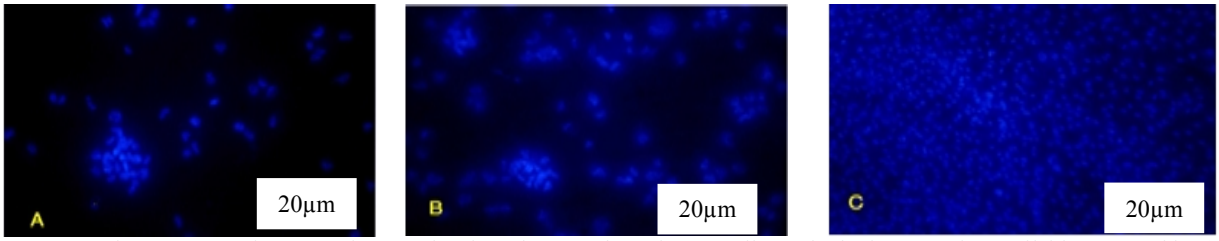

Figure 7. Fluorescence microscope image of Cultured mesenchymal stem cells on the hydroxyapatite scaffolds prepared by (a) NaOH, (b) PLPW and (c) Thermal

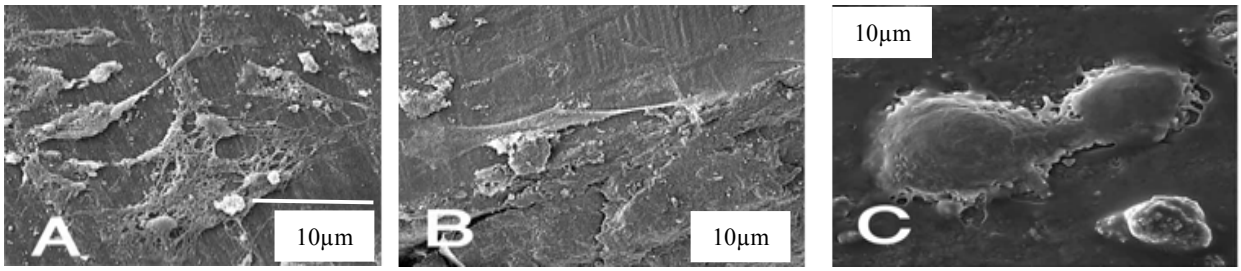

Figure 8. SEM of the mesenchymal stem cells on the hydroxyapatite scaffolds prepared by (a) NaOH (b) PLPW and (c) Thermal

4. CONCLUSION REMARKS

The all of thermal processing, pressurized low polarity water and alkaline hydrothermal processing have ability to leaving a pure hydroxyl carbonate apatite from raw bovine bone, but hydroxyl carbonate apatite obtained by PLPW process, leaving more carbonate contents and preferable for medical application. Carbonate groups in thermal calcinations are stable up to 750 °C. In range of 750 °C to 900 °C, all carbonate groups decomposes and over the 900 °C, pure natural hydroxyapatite is obtained. By increasing of temperature from 250°C for aqueous hydrothermal to 700°C for thermal processing, the crystallization degree of powders is alternatively increased. The umbilical cord mesenchymal stem cells could grow in all scaffolds. However, the survival was higher in the cells cultured on HCAp obtained by thermal calcinations.

5. ACKNOWLEDGMENTS

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Comparison of Purity and Properties of Hydroxyl Carbonate Apatite Extracted from Natural Thigh Bone by Different Physiochemical Methods

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چکیده

خانواده ترکیبات بر پایه آپاتیت دارای قابلیت های متعددی همچون زیست سازگاری، زیست فعالی، استخوان سازی، سمی نبودن و همچنین ایجاد خاصیت غیر تدافعی در بدن می باشند بطوریکه باعث شده است این ماده به عنوان یک بیوسرامیک در کاربردهای مختلف اورتوپدی و جراحی پیوند استخوان به عنوان جایگزین، ترمیم کننده و انواع اتصالات به طور گسترده ای مورد استفاده قرار گیرد. استخوان طبیعی موجودات زنده یکی از منابع مهم و در دسترس بوده که می تواند برای تولید انواع ترکیبات آپاتیت مورد استفاده قرار گیرد. در این کار تحقیقاتی، هیدروکسی کربنات آپاتیت کربناته طبیعی از استخوان زائد گاو از سه فرآیند متفاوت عملیات حرارتی کلسینه (در دمای ۷۰۰ °C)، تجزیه هیدروترمال قلیایی (۲۷۵ °C) و تجزیه هیدروترمال آبی (۲۵۰ °C) استخراج شده است. نمونه هایی از پودر خام استخوان مورد آنالیز حرارتی افتراقی (DTA) و آنالیز حرارتی وزنی (TG) قرار گرفته است. پودر بدست آمده از فرآیند های فوق مورد شناسایی توسط آنالیز طیف سنجی مادون قرمز (FTIR)، پراش اشعه ایکس (XRD) و مشاهدات میکروسکوپ الکترونی روبشی (SEM) قرار گرفته است. برای ارزیابی زیست فعالی پودر بدست آمده آزمایشات کشت سلولی در محیط محلول شبیه سازی شده بدن انجام گرفت. نتایج بدست آمده همگی دلالت بر قابلیت خوب حذف ترکیبات آلی از جمله کُلاژن های در درون استخوان گاو و تولید هیدروکسی کربنات آپاتیت طبیعی میکرو و نانو ساختار با بازدهی تا ٪۶۰ از سه روش فوق بخصوص روش هیدروترمال آبی دارند. با انجام عملیات حرارتی کلسیناسیون عموماً تمام ترکیبات آلی تا دمای ۶۰۰ °C از ساختار خارج شده و گروه کربنات در ساختار کریستالی در کنار یون (OH^-) باقی می ماند. با افزایش دما در بالاتر از ۹۰۰ °C گروه کربنات به طور کامل از ساختار خارج گردیده و هیدروکسی آپاتیت خالص کریستالی حاصل می شود. نتایج کشت سلولی نشان دهنده قابلیت بالاتر زیست فعالی برای آپاتیت کربناته نسبت به هیدروکسی آپاتیت از لحاظ بیولوژیکی برای کاربردهای اورتوپدی و بیوسرامیکی می باشد بطوریکه فیلم رسوبی چسبنده و پایدار بر روی دارپست هیدرو آپاتیت کربناته شکل می گیرد.

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