Published in final edited form as:

J Craniofac Surg. 2013 May ; 24(3): 1042–1045. doi:10.1097/SCS.0b013e318290258b.

Hydroxyapatite synthesis using EDTA

Nak Heon Kang, M.D.¹, Soon Je Kim, M.D.¹, Seung Han Song, M.D.^{1,*}, Sang mun Choi, M.D.¹, Sik Young Choi, Ph.D.², and Youn Jung Kim, Ph.D.²

¹Department of Plastic & Reconstructive Surgery, College of Medicine, Chungnam National University, Daejeon, Korea

²Department of Applied Chemistry, Andong National University, Andong, Korea

Abstract

Bone comprises structure of body and is consisted of inorganic substances. It exists in an organic structure in the body. Even though it is firm and has self healing mechanism, it can be damaged by trauma, cancer, or bone diseases. Allograft can be an alternative solution for autologous bone graft.

Hydroxyapatite($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), an excellent candidate for allograft, can be applied to bone defect area. There are several methods to produce hydroxyapatite, however economical cost and time consuming make the production difficult. In this study we synthesized the hydroxyapatite with Ethenediamine tetraacetic acid. Freeze Dried Bone Allograft(Hans Biomed) was used to be a control group.

Synthesized hydroxyapatite was a rod shape, white powdery type substance with 2 ~ 5 μm length and 0.5 ~ 1 μm width. X-ray diffraction showed the highest sharp peak at 32° and high peaks at 25.8°, 39.8°, 46.8°, 49.5°, and 64.0° indicating a similar substance to the freeze Dried Bone Allograft. 3 days after the cell growth of synthesized hydroxyapatite showed 1.5 fold more than the Bone Allograft. Cellular and media alkaline phosphate activity increased similar to the bone alloagraft.

In this study we came up with a new method to produce the hydroxyapatite. It is a convenient method that can be held in room temperature and low pressure. Also the the product can be manufactured in large quantity. It can be also transformed into scaffold structure which will perform a stronger configuration. The manufacturing method will help the bony defect patients and make future medical products.

Keywords

EDTA; Hydroxyapatite

I. Introduction

Bone comprises the structure of the body and is consisted of inorganic substances. It exists in an organic structure in the body.¹ The core mineral and chemical structure of a bone consists of hydroxyapatite, collagen, fibrin, phosphorus and calcium. Even though it is firm

Address correspondence: Seung Han Song, M.D., Department of Plastic & Reconstructive Surgery, Chungnam National University Hospital, 640 Daesa-dong, Jung-gu, Daejeon, 301-721 Korea. Tel; 82-42-280-7386 / Fax: 82-42-280-7384 / silverwine78@cnuh.co.kr.

Ethical Adherence : We must obey general ethical adherence and this study does not has any financial or institution purpose

Conflicts of Interest and Source of Funding: All authors in our manuscript have no relationship any other finance, consultant, institution.

and has self healing mechanism, it can be damaged by trauma, cancer, or bone diseases. Currently, autogenous bone grafts and allografts are used to treat patients. However, an autogenous bone graft has limitations of quantity of donor area as well as morbidity of donors and when an allograft takes place, there is a possibility of morbidity of infectious disease and immune reaction. In case of absence of donor area and deterioration of patient's condition, usage of biomaterial can be considered.

As a principal component in inorganic substance of teeth and bones found in vertebrates, hydroxyapatite is used in its powder condition to supplement defective part of bones as filling material. When grafted, tissues are deposited which creates biocompatibility that enables multiplication.² At present, hydroxyapatite is produced in a sintered body of compact substance or a madreporite and numerous clinical trials are performed with it as graft material.

Generally, many methods are known in synthesizing hydroxyapatite such as sedimentation method, hydrolysis, hydrothermal method and co-precipitation.³ It takes tremendous financial cost and time in synthesizing pure hydroxyapatite which creates limitations in producing it in large quantities. In this study we synthesized the hydroxyapatite with Ethylenediamine tetraacetic acid. Freeze Dried Bone Allograft(Hans Biomed) was used to be a control group.

II. Materials and Methods

A. Synthesis and analysis of hydroxyapatite

Reagents, which were used in hydroxyapatite synthesis, are calcium nitrate tetrahydrate ($\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, Duksan Chemical), potassium phosphate (KH_2PO_4 , Duksan Chemical), ethylenediamine tetraacetic acid (EDTA, Duksan Chemical), and sodium hydroxide (NaOH, Duksan Chemical). To synthesize, 2-M of sodium hydroxide was dissolved in 0.25-M of EDTA and 0.25-M of calcium nitrate tetrahydrate was first dissolved. Then, 0.15-M of potassium phosphate was added to be completely dissolved in the solution. Suspended matter was filtered as glass fiber paper and went through reaction for 8 hours at 100°C. White sediments in the solution were washed with distilled water until it reached pH 8 and below, then after an hour of heat treatment at 600°C, white powder was obtained. Freeze dried bone allograft(FDBA) that are currently used for medical purposes were used as a control group. A crystal structure of a synthetic product and the control group were analyzed with X-ray diffractometer (D/MAX RAPID-S, Japan) using Cu Target (40 kV, 30 mA). The shape of the crystal was magnified 2000 times to be observed using scanning electron microscope. A specific surface area was first degassed for 8 hours at 200°C to be analyzed using BET surface area analyzer (ASAP-2010, USA). To analyze element contents in inorganic substance, X-ray fluorescent analyzer (ZSX PrimusII, Japan) was used.

B. Cell experiment and analysis method

1) In vitro cell culture—A synthesized product and the control group was produced into pellet shape of 13 mm ϕ × 2 mm(H) and was heated for 2 hours at 1,250°C ready to be used.

In order to see the growth of each specimen, osteoblast (MC3T3-E1) was used.

In the experiment, each specimen was attached to the bottom of 24-well cell culture plate.

To make the culture fluid, 10% of Fetal Bovine Serum(FBS, Gibco) and 1% of Penicillin streptomycin solution(JBI) were blended into cell culture media(α -MEM, Gibco).

The cells were dispensed into 2×10^4 cells/ml and were cultured for 3 days at 37°C in 5% CO₂ atmosphere. Cultured osteoblastic cells were washed 2 times with phosphate buffered saline(PBS, Gibco) and was separated using a centrifuge. Centrifugation had duration of 1 minute at 1,500 rpm. Separated cells were diluted in culture fluid and total number of cells per ml was calculated using Hemacytometer(USA).

2) A method for measurement of alkaline phosphatase (ALP) activity—Since p-nitrophenyl phosphate(pNPP) is hydrolyzed by alkaline phosphatase producing p-nitrophenol, ALP activity can be measured indirectly by measuring the amount of p-nitrophenol to compute ALP concentration. At this time, since number of cells can affect the degree of ALP activity, total amount of protein was measured to calculate the degree of ALP activity per cell units.

In this experiment, MC3T3-E1 cells were dispensed to 24-well plate where each specimen was cultured, creating concentration of 2×10^4 cells/well and cultured for 3 days. Then, 10% of FBS, 1% of antibiotics and 50 µg/ml of ascorbic acid replaced the existing culture medium.

It was cultured every 3 days until day 12 when culture fluid was removed and washed with PBS, then lysis buffer (0.02% Triton X-100 / 0.9% NaCl) was added to decompose cells with radiofrequency. The cells were then centrifuged for 15 minutes at 12,000 rpm, after which the liquid in the upper layer was selected to measure ALP activity.

300 µl of a cell solution, 500 µl of 1 M Tris-HCl buffer (pH 9.0), 100 µl of 5 mM MgCl₂ and 100 µl of 5 mM p-nitrophenyl phosphate (PNPP, Sigma, USA) were mixed into 1.5 ml tube for 30 minutes of reaction at 37°C and colorimetric test was performed at 405 nm.

Protein concentration was measured with Bradford method using bovine serum albumin (BSA) as a standard sample.

For statistical analysis of the data, Sigma Plot software (v8.0, SPSS) was used.

All results were indicated as average $\pm$ standard error (SE) and tests of significance were performed for each sample using one-way ANOVA test and Turkey's HSD test. Level of statistical significance (p-value) was determined as $P < 0.05$.

III. Results

A. Observation of hydroxyapatite

Freeze dried bone allograft which is the control group, did not have porosity formation in its external appearance and the shape of its crystal was not observed. The synthesized hydroxyapatite was a rod shape, white powdery type substance with 2 ~ 5 µm length and 0.5 ~ 1 µm width. It mostly has uniform crystal form and the tip of crystal is needle-shaped. Also, in the process of forming the synthesized product, crystal grains were not formed solely but they grew in the form of many rods. Just as results from scanning electron microscope, the the control group appeared in the form of chips without porosity formation either internally or externally with specific surface area of 0.18 m²/g. The synthesized product had the specific surface area of 4.74 m²/g, was formed with powder size of few µm, without porosity formation taking place inside the crystal grains(Fig. 1).

B. X-ray diffraction analysis of hydroxyapatite

The analysis of the the control group and the synthesized product using X-ray diffraction shows that the synthesized product had sharper crystal peak compared to the the control

group (Fig. 2). 2θ value showed the strongest peak at 32.0° . X-ray diffraction showed the highest sharp peak at 32° and high peaks at 25.8° , 39.8° , 46.8° , 49.5° , and 64.0° indicating a similar substance to the freeze Dried Bone Allograft.

From analysis of the synthesized product using JCPDS (powder diffraction standards) CARD, the synthesized product was identified as hydroxyapatite.

From analysis using X-ray diffraction, the the control group had higher contents of sodium, magnesium and silicon component compared to that of the synthesized product (Table 1). This implies that the the control group, as freeze dried bone allograft, had slightest amount of mineral nutrients remained that are needed for its biological activity.

C. The result of cell growth in vitro

3 days after the cell growth of synthesized hydroxyapatite showed 1.5 fold more than the Bone Allograft. Cellular and media alkaline phosphate activity increased similar to the bone allograft. Therefore, this research shows that synthesized hydroxyapatite has more outstanding results in osteoblast adhesion and in multiplication rate than freeze dried bone allograft that are sold for medical purposes (Fig. 3).

D. Measurement of alkaline phosphatase (ALP) in vitro

1) Alkaline phosphatase in cells—Over 12 days, amount of ALP changes were observed in developed osteoblasts on the surfaces of synthesized hydroxyapatite and of freeze dried bone allograft that are sold for medical purposes. The result indicated that for 3 days from the day 0 where osteoblasts alone were growing, synthesized hydroxyapatite showed relatively high ALP value and every 3 days the ALP value increased by 2–3 times. (Fig. 4).

After 12 days have passed, the value was 25% higher for synthesized hydroxyapatite than for freeze dried bone allograft used in medicine, and the difference was statistically significant ($P < 0.05$). The research shows that synthesized hydroxyapatite had higher activity of osteoblast than for freeze dried bone allograft used in medicine, therefore a better alternative.

2) Alkaline phosphatase in culture fluid—From observing ALP amount change in osteoblast culture fluid of synthesized hydroxyapatite and of freeze dried bone allograft used in medicine for a period of 12 days, there was no noticeable difference to day 6. However, from day 9, synthesized hydroxyapatite had high ALP value, creating a statistically significant difference ($P < 0.05$).

Such phenomenon created the same result as result from analyzing ALP in osteoblast cells, made possible by cells with highly active cells producing relatively large amount of active phosphorus which caused high value of ALP value in culture fluid (Fig. 5).

IV. Discussion

Hydroxyapatite is one of mineral components in apatite that exists in the nature and is written as $\text{Ca}_5(\text{PO}_4)_3(\text{OH})$, but generally used as $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$.

OH^- ion is converted with fluoride or chloride and become either fluoroapatite or chloroapatite.⁴ More than 50% of bone's component is made of converted structures of hydroxyapatite's inorganic substance.

Recently, hydroxyapatite produced to apply on exterior of metal implants that are inserted into body in order to change properties of the surface.

Hydroxyapatite applied on the surface decreases reaction to foreign substance in the body which could decrease complications after surgeries.

It can also be produced into many forms such as powder, beads and porous blocks. When part of bone is removed due to diseases as osteomyelitis, injury and cancer, scaffold formed of hydroxyapatite can be implanted around bone defects to induce formation of bone and to make bone graft easier.

There are number of ways to synthesize hydroxyapatite such as drying method, wetting method, hydrothermal method and sedimentation method.⁵ In drying method, controlling proportion ratio in composition is made easy but the method has flaws in characteristics of pulverulent body. Wetting method creates non-stoichiometric compound with slight difference in its initial and final product's composition but produces fine and even pulverulent body. Hydrothermal method produces pulverulent body with exceptional crystal but makes it difficult to produce fine grains.

The elemental powder produced by sedimentation method is most general economically. If hydroxyapatite powder is produced using sedimentation method, heat decomposition occurs in temperature higher than about 800°C in most cases, decomposing into hydroxyapatite, the stoichiometric composition without any calcium defects and into β -TCP(Tri-Calcium Phosphate). However, when temperature gets close to about 900°C, sintering process occurs in hydroxyapatite and can be a negative influence in sintering process.

To overcome such limitations in this study, unlike in other studies, pH level of 13 or higher was reached in the normal temperature and was able to produce uniform crystalline hydroxyapatite. The synthesis process was relatively easy and uniform crystal was observed with scanning electron microscope which makes it easier to produce into various scaffold to enable a role as bone substitute of a good quality(Fig. 1).

Also, the analysis of the synthesized product using JCPDS (powder diffraction standards) CARD showed the peak of hydroxyapatite and not any other peak, which means that it was synthesized into hydroxyapatite with a high degree of purity.

Peak in the control group was not sharp, since freeze dried bone allograft contains a large quantity of organic substance caused by biological activity although it is composed of hydroxyapatite.

There are two ways to study reactivity of biologically activated substance with a human body.

In vivo test is where material is inserted into a body and reactivity for a certain period of time is measured. In vitro test is where similar environment is created for the material and the degree of reactivity is measured in the environment.⁷ In this study, in vitro test was performed to study biological reactivity. Similar environment used in the experiment was mixed with cell culture media(α -MEM, Gibco) and Fetal Bovine Serum. After the graft, cell multiplication rate and ALP in cellular and media were measured. It is considered that the synthesized product has higher sodium(NA) than the control group because in its synthetic process, sodium was not completely removed but left residues (Table 1).

The synthesized product contained more phosphorus, which is the major component of hydroxyapatite, and the the control group had more calcium.

The control group had higher Ca/P mol rate which indicates the ratio between calcium and phosphorus and this shows that the the control group contains higher relative level of calcium than the synthesized product.⁸

In cell multiplication that took place for 3 days, the synthesized hydroxyapatite had cell multiplication of 1.5 times more than the the control group. This indicates that when grafted into defected bone, hydroxyapatite will have higher increase of osteoblasts compared to the existing medical materials.

From observing the change in ALP in osteoblasts for 12 days, the result showed a high ALP value in the synthesized product and the result was similarly high in observations of ALP changes in osteoblasts' cultural fluid for 12 days. This shows that the synthesized hydroxyapatite, compared to freeze dried bone allograft, is more effective in improving activity in developed osteoblasts which in turn increases graft success rate when used in actual allografts.

In this study we came up with a new method to produce the hydroxyapatite. It is a convenient method that can be held in room temperature and low pressure. In comparative experiment with freeze dried bone allograft that are used in medicine, similar result was obtained with existing products in terms of cell culture and ALP change over 12 days.

The result shows that hydroxyapatite is effective in activating developed osteoblasts to a greater degree and promote bone synthesis. We have increased life expectancy and out of many materials that can substitute bones, hydroxyapatite that largely contains component of a bone, is considered as a promising solution. Also the the product can be manufactured in large quantity. It can be also transformed into scaffold structure which will perform a stronger configuration. The manufacturing method will help the bony defect patients and make future medical products.

References

1. Carlos L, Julie M. Ceramic Bulletin. 1991; 70:95.
2. Son WG, Lee YH. J Korean Soc Plast Reconstr Surg. 2004; 31:649.
3. Jarco M, Bolen CH. J Mater Sci. 1976; 11:2027.
4. Carlos, J.; José, C.; Foltin, J. Basic Histology, Text & Atlas. 10. Vol. 144. New York: McGraw-Hill; 2003.
5. Chun SY, Kim HK, Lee KH, Lee BH. J Korean Ceramic Soc. 1993; 30:215.
6. Takafumi, K. Inorganic Phosphate Materials. Vol. 79. Elsevier; Amsterdam: 1989. Calcium Orthophosphates.
7. Lim BI, Choi SY, Jung HJ. J Korean Ceramic Soc. 1996; 33:1003.
8. Lee SK, Ko HY, Lee GJ. J Korean Ceramic Soc. 1989; 26:171.

Fig. 1.
Morphology and size for the control group(Left) and the synthesis group(Right).

Fig. 2.
X-ray diffractometer for the control group and the synthesis sample

Fig. 3.
Cell growth after 3 days of proliferation. The synthesis group showed 1.5 fold higher than the control group.

Fig. 4. Cellular ALP activity of 12 days between the synthesized Hydroxyapatite and the Freeze Dried Bone Allograft

Fig. 5.
Medium ALP activity of 12 days between the synthesized Hydroxyapatite and the Freeze Dried Bone Allograft

Table 1

The percentage of components for solids obtained from the control group and the sample.

Components (%)	NaO	MgO	SiO2	P2O5	K2O	CaO
Samples						
Control group	1.07	0.53	1.35	36.02	0.03	61.00
Synthesis sample	0.44	0.21	0.75	38.90	1.52	58.18