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BIOCOMPATIBILITY OF IN HOUSE β-TRICALCIUM PHOSPHATE CERAMICS WITH NORMAL HUMAN OSTEOBLAST CELL

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Abstract

β-Tricalcium Phosphate (β–TCP) have been widely used as an implant materials. It has been successfully produced locally using two different method which is hydrothermal and precipitation. The aim of the study was to determine the biocompatibility of β-TCP prepared by hydrothermal and precipitation method with normal human osteoblast (NHOst) cells. For this purpose cytotoxicity of the material was assessed using an Alamar Blue method to determine the viability of NHOst cells grown with extracts of β-TCP in various concentrations. In addition NHOst grown on β-TCP ceramics were examined under an inverted microscope after 4, 24, 48 and 72 hours to verify cell attachment. Staining was done using Calcein AM and Ethidium homodimers to assess viability using a Confocal Laser Scanning Microscope (CLSM). The results showed that neither hydrothermal β-TCP nor precipitation β-TCP were cytotoxic with either of the method applied.

Keywords: β-Tricalcium phosphate, Cytotoxicity, Attachment, CLSM, Osteoblast.

1. Introduction

Biomaterials used in medical devices, regardless of whether they are permanent or biodegradable, naturally occurring or synthetic, need to be biocompatible. Biocompatible is the ability of a material to perform with an appropriate host response in a specific application [1].

Advances in biomaterial research now allow wide use of synthetic bone ceramic materials such as tricalcium phosphate, $Ca_3(PO_4)_2$. A biologically active biodegradable ceramic used for bone replacement. There are two types of TCP which are alpha (α) and beta (β) phases. The β-TCP phase is stable below 1125°C while the α phase is only stable in the range of 1125-1430°C. α-TCP is a very reactive and degrades rapidly in vitro, so it is not used as bone graft material and the present study therefore only focused on β-TCP [2]. β-TCP has been widely utilized clinically since 1970's as synthetic bone filler in the fields of dental and orthopedic surgery, and its biocompatibility has been confirmed in experimental and clinical trials [3].

Due to the broad range potential uses of the material, research has been done in School of Materials and Minerals Resources Engineering, Nibong Tebal, USM to produce the material locally. Synthesization of β-TCP has been achieved by two methods, namely hydrothermal and precipitation. Hydrothermal method is a wet-chemical process promoted under high-pressured water above 100°C while precipitation method is the most widely synthesization method used which involved the reaction of mixture under control condition of pH, temperature and atmosphere. The density of β-TCP obtained by the precipitation method is around 3.1 g/cm^3 (similar to that of commercial β-TCP (ρ=3.17 g/cm^3) whilst the hydrothermal method produces powders of higher density (around 3.7 g/cm^3).

One of the criteria for biocompatibility is that the material is not toxic to cells. For ideal in vitro tests, biomaterials should match the cell populations of implant sites. Normal Human Osteoblasts were chosen as a model cell for in vitro biocompatibility study because this β-TCP was intended to be used as bone implant materials. Cell attachment to the materials was also evaluate as it is the first step in the process of cell interaction and will affect next cellular response. In this study, the interaction between hydrothermal and precipitation β-TCP with NHOst was investigated to assess the biocompatibility of this locally produced biomaterial. For assessment of cytotoxicity, vital staining was carried out as proposed by the National Guidelines for cytotoxicity test ISO 10993.

2. Materials and Methods

2.1. Materials

The β-TCP ceramic for bone replacement was prepared by School of Materials and Minerals Resources Engineering, Nibong Tebal, USM using precipitation and hydrothermal method. *In vitro* testing for cytotoxicity was performed according to ISO 10993 [4]. Samples were sterilized in an autoclaved at 120°C for 20 minutes and three replicates were tested for the samples.

2.2. Preparation of extracts

After sterilization, β-TCP extracts were prepared by incubating the solid β-TCP with culture medium Osteoblast Growth Medium (OGM, Lonza) (OBM supplemented with Fetal Bovine Serum, GA-1000 and Ascorbic acid) for 48 hours at 37°C. To meet ISO requirements, the concentration of sample in extraction solution was 100 mg/ml. Dilutions were prepared by addition of OGM to the extracts in order to achieve concentrations of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml, respectively.

2.3. Cell culture

Normal human osteoblast cells (NHOst) obtained from Lonza, USA were cultured in OGM (OBM supplemented with Fetal Bovine Serum, GA-1000 and Ascorbic acid) in a standard CO_2 incubator at 37°C. The cells were used for experiment between passages 5 to 10. The behavior of cells on the samples was investigated by in vitro test cytotoxicity as described below.

2.4. Cytotoxicity determination with extracts

The cells were cultured first at cell density 5×10^3 cells/cm for 24 hours at 37°C in 96 well plates. The medium was replaced with sample extracts. A negative control was also used which was the cells without test material. The plates were incubated in a CO_2 incubator for 72 hours. Next, 10µl of alamar blue was added to all wells followed by further incubation for 4 hours. Finally an Elisa reader (TECAN) was used to read the absorbance at reference and test wavelengths of 600 nm and 570 nm. Cell viability percentages were calculated according to the following equation: % cell viability = [OD (sample) / OD (control)] × 100%. Alamar blue is a redox indicator that based on the ability of metabolically active cells to convert the reagent into a fluorescent and colorimetric indicator. Damage and non viable cells have lower innate metabolic activity and thus generate proportionally lower signals.

2.5. Cytotoxicity, cell attachment and vital staining with ceramics

A viability test using Calcein AM and Ethidium homodimer-1 (Molecular Probes, USA) was also used to quantify viable cell growth for cells seeded directly onto test materials. Calcein AM is cleaved by cellular esterases present within viable cells to form a fluorescent green product which is membrane impermeable. Ethidium homodimer-1 is a fluorescent red marker which binds to nucleic acids and only passes through the compromised membrane of non viable cells.

Hydrothermal and precipitation β-TCP were placed to 4 well plates and 0.5 ml of growth medium were added and pre-incubated for 30 minutes to humidify the materials. After that the medium were removed and the materials were seeded with 3×10^4 cells/ml placed on the material. After incubation at 37°C for 72 hours, cell response towards the material was verified after 4, 24, 48 and 72 hours under an inverted microscope. Images were captured using an inverted microscope (Zeiss) supplemented with Imagepro Software.

At the end of incubation period, the materials were rinsed 3 times in Phosphate Buffer Saline (PBS). Working reagent containing 10 µl of the supplied Calcein AM and 50 µl of Ethidium Homodimer stock in 5 ml PBS were added on materials and incubated 30 minutes at room temperature in darkness. Then the materials were taken out and mounted with antifade solution on slides. Stained cells were examined using Confocal Laser Scanning Microscope (Leica) under 100x magnification. Each image was printed and live (green) and dead (red) cells were counted. The percentage of live cells was calculated.

3. Results and Discussion

The prediction of the aerodynamic coefficients of the investigated projectiles shown in Fig. 1 was carried using the methods and the computer programme described above. The effects of forebody and afterbody shapes on the aerodynamics at supersonic speeds are analysed in this paper.

Synthetic bone graft materials are frequently used in orthopaedic and dental field recently due to its biocompatibility and ready availability. β-TCP is the promising bone graft as it have similar composition to human natural bone, osteoconductive and highly biocompatible. The advantage of β-TCP which has gained more attention is its biodegradability. After several period of implantation, β-TCP will degrade and will be replaced with natural bone tissue, which is good for remodelling phase during bone healing process.

The synthesization of both β-TCP at School of Materials and Minerals Resources Engineering utilizing the same starting material which were H_3PO_4 solution and $Ca(OH)_2$ solution. Both of these β-TCP were then subjected *to in vitro* cytotoxicity test using normal human osteoblast (NHOst) as a model cell. Osteoblast cells were chosen because it is bone forming cells which actively involved during entire bone regeneration process.

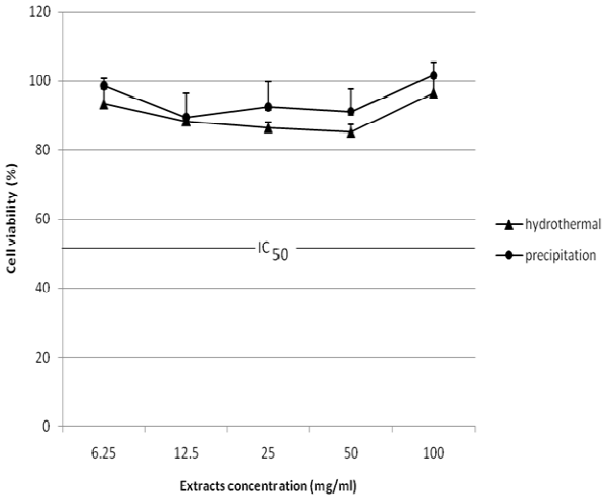

Fig. 1. Percentage of Cell Viability of Precipitation and Hydrothermal β-TCP Extraction in NHOst Using Alamar Blue Assay.

The cytotoxicity of β-TCP with osteoblast cells were evaluate using Alamar Blue assay. Figure 1 represents curves of the viability percentage of hydrothermal and precipitation β-TCP compared to control. From the figure, even at the highest concentration of β-TCP prepared by both methods did not show any evidence of cytotoxicity with the osteoblast cells. The IC_{50} (50% Inhibitory Concentration) endpoint were used to evaluate the cytotoxicity effects of the materials at different concentration applied. IC_{50} is define as half inhibitory concentration, which the concentration of materials to inhibit half of maximum biological process. The IC_{50} were used by many researchers to evaluate the cytotoxicity of their tested materials [5-7]. The material is considered as toxic if the material inhibits more than half of cells viability. According to the Fig. 1, there is no adverse effect concentrations were observed from the curves. Percentages of cell viability of the sample extraction in both materials were more than 90% indicates both hydrothermal and precipitation β-TCP is not toxic to the osteoblast cells.

Next, direct contact experiments were performed on β-TCP ceramics, using osteoblast in order to evaluate the cell/materials interaction with respect to cell attachment and proliferation. Figure 2 indicates that the increased cell density is seen near both materials similar to control. The black area is a material and cells were able to grow along the materials. The cells were reached confluent at the end of 72 hours incubation and did not show any cytophatic morphology during entire period. These interactions show the osteoconductive properties of the β-TCP and the results were in agreement with other researchers that have been conducted test to evaluate the interactions of β-TCP with osteoblast cells [8-11].

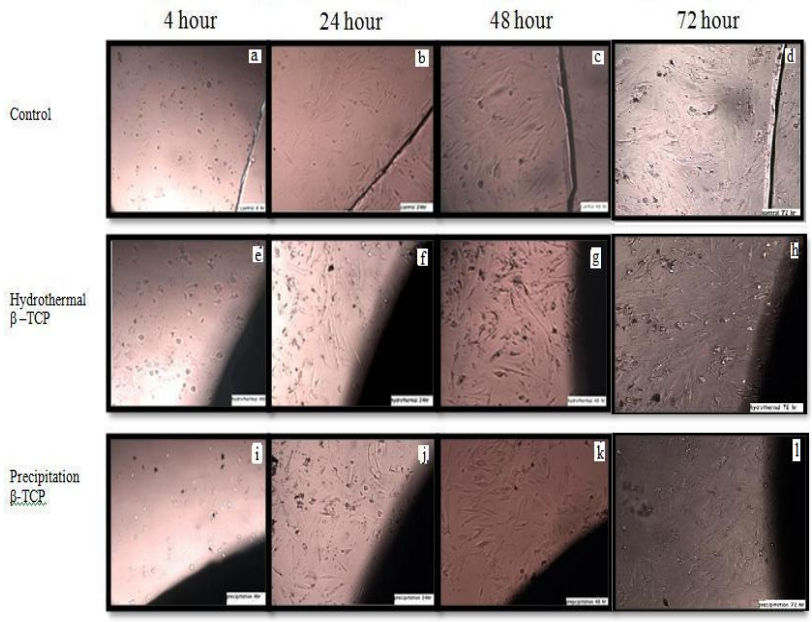

Fig. 2. Image of Cell Attachment near the Material under Inverted Microscope Captured Using Imagepro Software at 4, 24, 48 and 72 Hours of Incubation (100x magnification).

The viability and attachment of osteoblast cells seeding directly onto surface of a material were then evaluated using Confocal Laser Scanning Microscope. The cells were stained using Calcein AM and Ethidium homodimer that gave fluorescent green color to the viable cell and fluorescent red color to the non viable cell. Result in Fig. 3 showed the cells were viable and able to attach on the material surface. The ability of cells to attach and spread on the materials is important phenomena which showing the materials surface is absent from toxic materials and favor for cell growth. Cell line is very sensitive to any changes, therefore if any toxic compound is present surround them, cell will not attach and will exhibit the zone of inhibition around the materials or maybe die. When a material introduce to the human body, cells-materials interaction will occur. Research that have been done by Kondo et al., showed highly purified β-TCP has a good biocompatibility when implanted *in vivo* since bioresorption and bone formation started at an early stage after implantation [12].

Osteoblast is an anchorage-dependent cell. Thus the ability of these cells to be attached to implant materials is important for its survival. Attachment is the first step of interaction between cells and materials and this attachment quality will influence the ability of cells to proliferate and differentiate when incorporated with the implant [13-15]. Images of viable cells attachment onto both tested materials showed clear evident that both hydrothermal and precipitation β-TCP were not toxic to the cells.

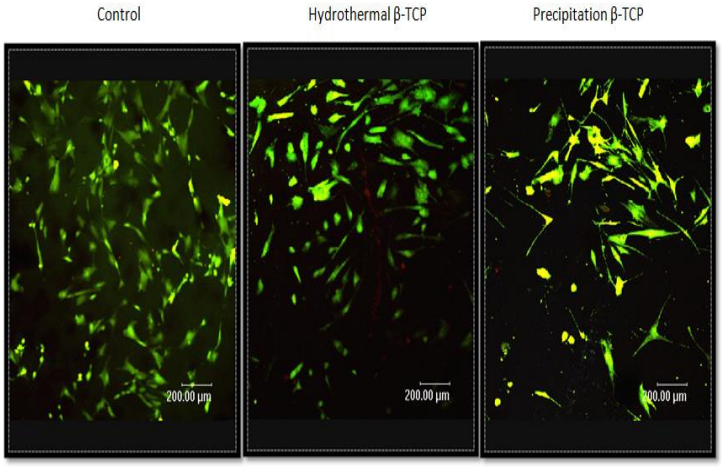

Fig. 3. Image of Stained Cells Grown onto Material Viewed Using Confocal Laser Scanning Microscope. Dead Cells were Stained Red and Live Cells Were Stained Green (Bar = 200 µm).

All the results showed that the hydrothermal and precipitation β-TCP posses excellent biocompatibility properties in this study. Both of tested materials shows the lack of cytotoxicity activity, and ability to support cells attachment and growth. As new locally produced materials, this finding gives the valuable knowledge of materials performance in order to produce the ideal biomaterials. Ideal biomaterials must not only have the excellent mechanical properties but also gives the excellent biocompatibility during the incorporation into the implant sites. Ready availability

and cheaper production cost is another factor that is important during the production of biomaterials. Hence, the best synthesization method which does not compromise the biomaterials performance must be achieved.

In-vitro test is important for initial screening of material which is simple, quick to perform, less expensive and can be controlled. In order to evaluate the overall biocompatibility of biomaterials, further test need to be performed such as *in-vivo* and clinical test. The results provided evidence that the material is not cytotoxic to the osteoblast cells, however further study is needed to fully understands the biocompatibility behavior of hydrothermal and precipitation β-TCP.

4. Conclusions

The results demonstrated that β-TCP produced by either hydrothermal or precipitation appeared to be cytocompatible under our experimental condition. Osteoblast cells shows non cytotoxicity response when exposed to the both β-TCP extracts at various concentration applied. Osteoblast cell were able to attach and viable on both hydrothermal and precipitation β-TCP. There is no differences between both hydrothermal and precipitation β-TCP in term of *in vitro* cytotoxic evaluation.

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