UNIVERSITI SAINS MALAYSIA
GERAN PENYELIDIKAN UNIVERSITI
PENYELIDIKAN
LAPORAN AKHIR

CYTOTOXICITY EVALUATION OF TRICALCIUM PHOSPHATE ON HUMAN OSTEOBLAST CELL LINE

PENYELIDIK

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PENYELIDIK BERSAMA

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NOR SHAMSURIA OMAR

2012

UNIVERSITI SAINS MALAYSIA

LAPORAN AKHIR PROJEK PENYELIDIKAN JANGKA
FINAL REPORT OF SHORT TERM RESEARCH PROJECT
Sila kemukakan laporan akhir ini melalui Jawatankuasa Penyelidika
Pengajian dan Dekan/Pengarah/Ketua Jabatan kepada Pejabat Pelan

1. **Nama Ketua Penyelidik:** **Dr Dasmawati Mohamad**
   Name of Research Leader

   ☐ Profesor Madya/ *Assoc. Prof.* ☑ Dr./ *Dr.* ☐ Encik/Puan/Cik *Mr/Mrs/Ms*

2. **Pusat Tanggungjawab (PTJ):**
   School/Department **School of Dental Sciences**

3. **Nama Penyelidik Bersama:**
   Name of Co-Researcher **Prof. Radzali Othman, Nor Shamsuria Omar**

4. **Tajuk Projek:**
   Title of Project **Cytotoxicity Evaluation of Tricalcium Phosphate on Human Osteoblast Cell Line**

5. **Ringkasan Penilaian**/*Summary of Assessment*:	Tidak Mencukupi *Inadequate*		Boleh Diterima *Acceptable*	Sangat Baik *Very Good*	
	1	2	3	4	5
i) **Pencapaian objektif projek:** *Achievement of project objectives*	☐	☐	☐	☑	☐
ii) **Kualiti output:** *Quality of outputs*	☐	☐	☐	☑	☐
iii) **Kualiti impak:** *Quality of impacts*	☐	☐	☐	☑	☐
iv) **Pemindahan teknologi/potensi pengkomersialan:** *Technology transfer/commercialization potential*	☐	☐	☑	☐	☐
v) **Kualiti dan usahasama :** *Quality and intensity of collaboration*	☐	☐	☐	☑	☐
vi) **Penilaian kepentingan secara keseluruhan:** *Overall assessment of benefits*	☐	☐	☐	☑	☐

6. Abstrak Penyelidikan
(Perlu disediakan di antara 100 - 200 perkataan di dalam **Bahasa Malaysia dan juga Bahasa Inggeris.** Abstrak ini akan dimuatkan dalam Laporan Tahunan Bahagian Penyelidikan & Inovasi sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada pihak Universiti & masyarakat luar).

Abstract of Research
(An abstract of between 100 and 200 words must be prepared in Bahasa Malaysia and in English).
This abstract will be included in the Annual Report of the Research and Innovation Section at a later date as a means of presenting the project findings of the researcher/s to the University and the community at large)

The aim of the study was to determine the biocompatibility of β-Tricalcium Phosphate (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.

Objektif kajian ini adalah untuk menguji biokeserasian β-Trikalsium Fosfat (β-TCP) yang telah disintesis menggunakan dua kaedah yang berlainan iaitu proses hidrotermal dan proses pemendakan dengan sel osteoblast manusia normal (NHOst). Kesitotoksidan bahan di analisa melalui kaedah Alamar Biru untuk melihat kebolehidupan sel membesar didalam pelbagai kepekatan larutan β-TCP. NHOst yang dibesarkan/dibela diatas seramik β-TCP juga dilihat dibawah inverted mikroskop selepas 4, 24, 48 dan 72 jam untuk mengesahkan pelekatan sel. Calcein AM dan Ethidium homodimers dilumurkan untuk melihat kebolehidupan sel menggunakan Confocal Laser Scanning Microscope (CLSM). Hasil ujian menunjukkan kedua-dua β-TCP yang dihasilkan melaui dua kaedah yang berbeza iatu proses hidrotermal dan proses pemendakan tidak toksid.

7. Sila sediakan laporan teknikal lengkap yang menerangkan keseluruhan projek ini.
[Sila gunakan kertas berasingan]
Applicant are required to prepare a Comprehensive Technical Report explaning the project.
(This report must be appended separately)

Senaraikan kata kunci yang mencerminkan penyelidikan anda:
List the key words that reflects your research:

Bahasa Malaysia	*Bahasa Inggeris*
Biokeserasian	Biocompatibility
β-Trikalsium Fosfat (β-TCP)	β-Tricalcium Phosphate (β-TCP)
pelekatan sel	cell attachment

8. Output dan Faedah Projek
Output and Benefits of Project

(a) * **Penerbitan Jurnal**
Publication of Journals
(Sila nyatakan jenis, tajuk, pengarang/editor, tahun terbitan dan di mana telah diterbit/diserahkan)
(State type, title, author/editor, publication year and where it has been published/submitted)

1. Nurshuhada Mohd Nazir, Dasmawati Mohamad, Md. Azman Seeni Mohamad, Nor Shamsuria Omar, Radzali Othman, (2010). "Biocompatibility Of In House B-Tricalcium Phosphate Ceramics With Normal Human Osteoblast Cell". World Engineering Congress (WEC) 2010 Proceeding, Kuching, Sarawak. 2-5 Aug 2010. ISBN: 978-983-4357-19-1, pp. 261-265
2. Nurshuhada Mohd Nazir, Dasmawati Mohamad, Md. Azman Seeni Mohamad, Nor Shamsuria Omar, Radzali Othman, (2011). In-Vitro Cytotoxic and Genotoxic Evaluation of In-House β-Tricalcium Phosphate Ceramics. Biomaterials 2010, Proceeding of Regional Biomaterial Scientific Meeting (RBSM 2010), pg 93-97. ISBN: 978-983-44364-2-1
3. Nurshuhada Mohd Nazir, Dasmawati Mohamad, Md. Azman Seeni Mohamad, Nor Shamsuria Omar, Radzali Othman, (2012). "Biocompatibility of in house β-Tricalcium Phosphate ceramics with normal human osteoblast cell". Journal of Engineering Science and Technology (JESTEC), Vol 7(2), pg 169-176. SCOPUS

(b) **Faedah-faedah lain seperti perkembangan produk, pengkomersialan produk/pendaftaran paten atau impak kepada dasar dan masyarakat.**
State other benefits such as product development, product commercialisation/patent registration or impact on source and society.

-

* Sila berikan salinan/*Kindly provide copies*

(c) **Latihan Sumber Manusia**
Training in Human Resources

i) Pelajar Sarjana: Nurshuhada Mohd Nazir ✓

Graduates Students
(Perincikan nama, ijazah dan status)
(Provide names, degrees and status)
MSc Research Mode .
Status- Submission of thesis ✓

ii) Lain-lain:
Others

9. Peralatan yang Telah Dibeli:
Equipment that has been purchased

-

Komen Jawatankuasa Penyelidikan Pusat Pengajian/Pusat
Comments by the Research Committees of Schools/Centres

This research project was successfully completed with one MSc research mode graduate and a number of publications. The objectives have generally been met within the time frame and budget constraints.

Comprehensive Technical Report

This study utilized the β-TCP ceramic for bone replacement which was prepared by School of Materials and Minerals Resources Engineering, Nibong Tebal, USM using precipitation and hydrothermal method. General objective and specific objectives of the study were as below:

General Objective

1. To study the cytotoxicity of experimental ceramic bone replacement to the human cells.

Specific Objective

1. To determine the cytotoxic effect of experimental beta tricalcium phosphate (β-TCP) cement prepared by precipitation method on osteoblast cells in vitro.
2. To determine the cytotoxic effect of experimental beta tricalcium phosphate (β-TCP) cement prepared by hydrothermal method on osteoblast cells in vitro.
3. To evaluate the cell adhesion on TCP beta tricalcium phosphate (β-TCP) cement under Confocal Laser Scanning Microscopy

All the above objectives were successfully achieved within this study. The details of the study can be read as below.

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 in 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. Since α -TCP is a very reactive and degrades rapidly in vitro it is not used as bone graft material and the present study therefore obly focused on β-TCP [2].

This form has been widely utilized clinically since 1970's as an artificial 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, are precipitation and the hydrothermal. 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/cm3)) whilst the hydrothermal method produces powders of higher density (around 3.7 g/cm3).

One of the criteria for biocompatibility is that the material is not toxic to cells. For an ideal in vitro approach, biomaterials should match the cell populations typical of implant sites, which in both orthopaedic and dental cases are mainly Normal Human Osteoblasts (NHOst). Cell attachment to the materials is the first step in the process of cell interaction and will affect cellular responses. In this study, the interaction between hydrothermal and precipitation B-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, an Internationally accepted standard EN 30993 and American FDA's bluebook memorandum G95.

Experimental

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

In vitro testing for cytotoxicity was performed according to ISO 10993- part 5[4]. Samples were sterilized in an autoclaved at 120°C for 20 minutes and three replicates were tested for the samples and controls coverslips.

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 concentrationof sample in extraction solution was 0.2 g/ml. Dilutions were prepared by addition of OGM to the extracts to obtain concentrations of 3.125%, 6.25%, 12.5%, 25%, 50% and 100%, respectively.

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 passage 5 to 10. The behavior of cells on the samples was investigated by in vitro test cytotoxicity as described below.

Cytotoxicity determination with extracts

The cells were cultured first at cell density $5x10^3$ cells/cm for 24 hours at 37 °C in 96 well plate. The medium was replaced with sample extracts. A negative control was included was the cells without test material. The plates were incubated in a CO_2 incubator for 72 hours.

After that 10µl of alamar blue was added to all wells followed by further incubation for 4 hours. Finally an Elisa reader was used to read the absorbance at reference and test wavelengths of 600nm and 570nm. Cell viability percentages were calculated according to the following equation: % cell viability = [OD(sample)/OD (control)] x 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.

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.

Materials were placed to 4 well plates and 0.5ml 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 $3x10^4$ cells/ml placed on the material. After incubation at 37 °C for 72 hours, cell attachment to 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 50µl 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 percent of live cells = the number of live cells/ (the number of live cells + the number of dead cells) was calculated.

Results and Discussion

3.1 Cytotoxic evaluation

Figure 1 shows curves of the diluted extracts for two materials (hydrothermal and precipitation β-TCP) versus the percentage of formed colonies regarding to control NHOst. From these curves, it is possible to observed that the extracts with highest material concentration and

not cause death of the cells, indicating the cytotoxicity of the material. The sample extraction for both materials, presented percentage of cell viability superior to 100%. These high percentage, might have explained by some retardation in growth of the control if the control medium had not been change. In fact, all of the media were simultaneously changed, thus cell proliferation might be related to release of substances stimulating cell growth [4]. The biocompatibility of a biomaterials is affected strongly by the kind of compounds that it can release. Calcium and phosphate are the most physiological chemical species, being involved in the formation of the hydroxyapatite in both teeth and bones [5]. Therefore the leaching of these elements from β-TCP can generally be considered useful and beneficial.

Next, direct contact experiments were performed on β-TCP ceramics, using NHOst 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 the both materials similar to control. It was already possible to see the cell almost reached confluent at the end of 72 hours of incubation. However, the cells on the surface of hydrothermal β-TCP did not seem to be very well attached, when compared to the precipitation β-TCP and to the control as in Fig 3. In contrast, the cells seeded on the hydrothermal β-TCP are much better spread on the surface and the morphology look similar to the cells cultured on control coverslips. Figure 4 shows that the high viability percentage of attached cells among the counted cell in both hydrothermal and precipitation β-TCP. Even though the density of attached cells of both material is lower than control, but the viability was similar to that of the control. The cell attachment and proliferation onto materials were decreased with both hydrothermal and precipitation β-TCP when compared to control coverslips as in Fig 3. This might be due to the fact that the materials may adsorb some of constituents of the growth media including ion and protein, thereby essentially starving the

cells. A previous study reported that pre-incubation of this ceramic material with media reduces the interference and results in a corresponding increase in cell viability [6]. Another possible reason is the cell proliferation rate seems to be higher on control coverslip. Titanium is the best control to study cell attachment due to the fact that the cell proliferation rate onto titanium is almost similar to human body.

The results provided evidence that cytotoxicity is lacking with the material but further study is needed to fully understands the biocompatibility behavior of hydrothermal and precipitation β-TCP especially using molecular approaches.

Conclusion

The results demonstrated that β-TCP produced by either hydrothermal or precipitation appeared to be cytocompatible under our experimental condition. There is no significant difference of β-TCP producing by hydrothermal or precipitation method on in vitro test experiment.

References

[1] Williams DF (Ed.). Definitions in Biomaterials: Proceedings of a consensus Conference of the European Society for Biomaterials, Chester, England. Amsterdam: Elsevier, 1987.

[2] Radin S, Ducheyne P. The effect of calcium phosphate ceramic composition and structure on in vitro behavior.II. Precipitation. J Biomed Mater Res. 1993;27:139-43.

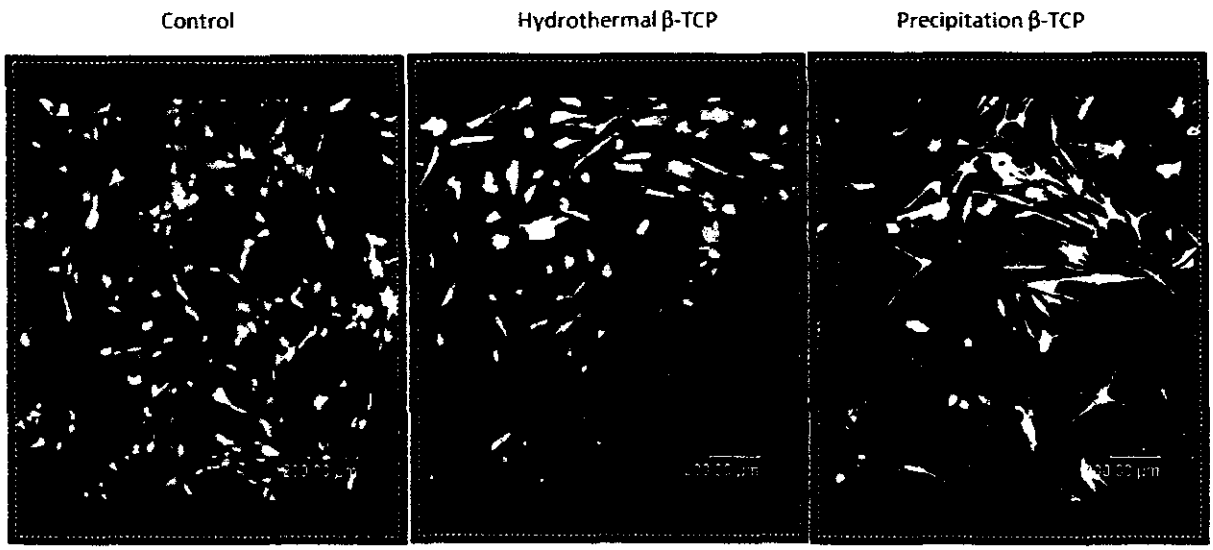

Fig. 3. Image of stained cells grown onto material viewed using Confocal Laser Scanning Microscope. Dead cells were stained red and live cells were stained green. (Bar = 200μm)

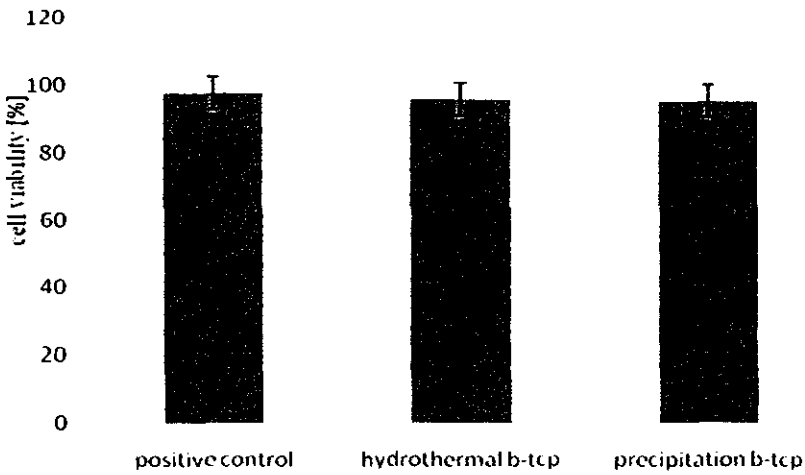

Fig. 4. Graph of percentage of cell viability of cells attached onto hydrothermal and precipitation β-TCP.

Publications (Proceeding/Conference)

1. Nurshuhada Mohd Nazir, Dasmawati Mohamad, Md. Azman Seeni Mohamad, Nor Shamsuria Omar, Radzali Othman, (2010). "Biocompatibility Of In House B-Tricalcium Phosphate Ceramics With Normal Human Osteoblast Cell". World Engineering Congress (WEC) 2010 Proceeding, Kuching, Sarawak. 2-5 Aug 2010. ISBN: 978-983-4357-19-1, pp. 261-265
2. Nurshuhada Mohd Nazir, Dasmawati Mohamad, Md. Azman Seeni Mohamad, Nor Shamsuria Omar, Radzali Othman, (2011). In-Vitro Cytotoxic and Genotoxic Evaluation of In-House β-Tricalcium Phosphate Ceramics. Biomaterials 2010, Proceeding of Regional Biomaterial Scientific Meeting (RBSM 2010), pg 93-97. ISBN: 978-983-44364-2-1
3. Nurshuhada Mohd Nazir, Dasmawati Mohamad, Md. Azman Seeni Mohamad, Nor Shamsuria Omar, Radzali Othman, (2012). "Biocompatibility of in house β-Tricalcium Phosphate ceramics with normal human osteoblast cell". Journal of Engineering Science and Technology (JESTEC), Vol 7(2), pg 169-176. SCOPUS

Development of Human Resources:

1. MSc (Research Mode) student: Nurshuhada Mohd Nazir