IN VITRO CYTOCOMPATIBILITY OF SYNTHETIC CALCIUM PHOSPHATE POWDER ON L929 FIBROBLAST CELL

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RINGKASAN: Dalam kajian ini, kesan dari serbuk dwifasa kalsium fosfat (BCP) sintetik terhadap keselserasian menggunakan sel kulit (fibroblas) L929 sebagai model in vitro telah dikaji dan dibandingkan dengan hydroxyapatit (HA) dan trikalsium fosfat (TCP) komersil. BCP disintesis menggunakan teknik sintesis basah pada nisbah Ca/P 1.6 dan dibakar dalam keadaan atmosfera untuk mendapatkan kedua-dua HA dan TCP pada masa yang sama. Hasil kajian menunjukkan bahawa semua bahan BCP, HA dan TCP adalah tidak toksik dan bioserasi pada sel L929. Tahap hidup sel asai Alamar Blue bagi kesemua bahan adalah melebihi 90 % pada semua kepekatan yang diuji. Butiran sel tidak berubah, tiada sel yang pecah dan tidak ada pengurangan pertumbuhan sel diperhatikan secara kualitatif menggunakan elusi MEM. Pemerhatian sel L929 yang dikultur bersama-sama BCP, HA dan TCP menunjukkan sel-sel hidup, membahagi secara baik, berkembang dan membiak dari hari pertama hingga hari ke 7 inkubasi. Ini menunjukkan bahawa BCP tidak toksik sebanding dengan HA dan TCP komersil. Keputusan ini menunjukkan bahawa BCP mempunyai ciri osteokonduktif, dan boleh memberi manfaat kepada aplikasi bioperubatan kerana kadar gabungan yang seimbang antara fasa lebih stabil (HA) dan lebih larut (TCP) dalam persekitaran tubuh.

ABSTRACT: The effects of a synthetic biphasic calcium phosphate powder (BCP) on cytocompatibility have been investigated and compared with the commercial hydroxyapatite (HA) and tricalcium phosphate (TCP) using L929 fibroblast cells as an in vitro model. BCP was synthesized using wet synthesis technique at Ca/P ratio 1.6 and sintered in atmospheric condition to obtain both HA and TCP peaks simultaneously. The results showed that all materials BCP, HA and TCP were non-toxic and biocompatible on L929 cells. Viability study using Alamar Blue assay indicates that cells treated with all materials were more than 90 % viable at all concentration tested. Qualitatively, the observation using MEM elution shows discrete intracytoplasmic granules, no cell lysis and no reduction of cell growth. Observations of L929 cells exposed to BCP, HA and TCP showed that the cells are viable and excellently differentiate. They were proliferating and growing well from day 1 to 7 of incubation. It is demonstrated that BCP cytotoxicity condition is comparable with commercial HA and TCP. These results suggest that BCP has osteoconductive property, and could be beneficial for biomedical application due to combination of a balanced rate between more stable (HA) and more soluble (TCP) phase in body environment.

KEYWORDS: calcium phosphate, cytocompatibility, non-toxic, osteoconductive

INTRODUCTION

Calcium phosphate (CaP) materials have received great interests since most of them are widely applied as biomedical materials, including bone fillers, bone tissue engineering scaffolds and bioactive coatings and composites due to their excellent biocompatibility, osteoconductive properties and similarity to the inorganic component of natural bone (Kaili *et al.*, 2014; Dorozhkin *et al.*, 2009; Xia *et al.*, 2013).

The most widely used CaP-based bioceramics are hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] and β-tricalcium phosphate [β-TCP, $Ca_3(PO_4)_2$]. HA is stable in a body fluid, while TCP is rather soluble. The in vivo and in vitro dissolution of CaP ceramics was found to be dependent on the composition, crystallinity, and pH of the solution (Soon-Ho *et al.*, 2003; Ducheyne *et al.*, 1993).

An important part of biomaterials research is the evaluation of biocompatibility in vitro under scientifically controlled conditions (Shektar *et al.*, 2009). Post synthesis in vitro cytocompatibility confirmation has become essential to comment on the potential use of materials in vivo. Fibroblasts are the predominant cells present in the loose connective tissues and are crucial for aiding wound repair mechanisms. Fibroblast cells adhering to their neighbours rather than the substrate cause fibrous encapsulation. Hence characterization and quantification of fibroblast cell viability on BCP material can strongly affirm their cytocompatibility and their potential use as an implant material. Fibroblasts also produce many essential components for the connective tissue, for instance, the extracellular components such as collagen, an aminoglycans in fibrous tissue. The attachment of fibroblast cells assists the integration of soft tissue with the implant, which infers improved vascularity at the implant surface and reduced chances of fibrous encapsulation and bacterial infection (Kalmodia *et al.*, 2010).

Our studies are focused on the in vitro cytocompatibility evaluations of BCP, HA and TCP. The evaluations include quantitative studies such as cell viability and qualitative study of MEM elution and cellular morphological observation using in vitro model, L929 mouse fibroblasts cells.

MATERIALS AND METHODS

Sample preparation

BCP was synthesized in-house by heating a mixture of calcium hydroxide (Fluka Analytical, Sigma-Aldrich Germany) and phosphoric acid (Avantor Material Performance, Thailand) with a Ca/P ratio of 1.6. After overnight stirring at 250 rpm, the apatite slurry was heat up to 85 °C and the temperature was maintained for 1 hr before cooling down to room temperature. The slurry was then ball milled for 1 hr and spray dried (GEA Niro; Mobile Minor 2000 H, Soeborg, Denmark) at 280 °C to convert it into apatite powder. The apatite powder was then sintered at 950 °C at 5 °C/min for 1.5 hrs (CMTS SC5 CM1148, Malaysia) to produce BCP that consists of HA and TCP powder. Commercially available HA powder (Captal 60 Plasma Biotal, UK) and TCP powder (Sigma-Aldrich, Germany) were used in this work. All powders consisted of pure phases as verified by X-Ray Diffraction, XRD (Bruker D8 Advanced, Germany).

Cell culture

L929 mouse subcutaneous connective tissue fibroblast cells (*Mus musculus*, NCTC clone 929, CCL-1™; ATCC, Manassas, VA, USA) were cultured in Dulbecco Modified Eagle's Medium (DMEM; Gibco® Invitrogen, Grand Island, NY, USA) containing penicillin/streptomycin (100/100 U; Gibco® Invitrogen), L-glutamine (200 mM; Gibco® Invitrogen), and HEPES (Gibco® Invitrogen) and supplemented with 10

% fetal bovine serum (Gibco® Invitrogen). They were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 (CO_2 incubator; Binder GmbH, Tutlingen, Germany). The medium was changed every 3 days under aseptic conditions.

Extraction preparation

BCP, HA and TCP extracts with a weight-to-volume ratio of 200 mg/ml were obtained by immersing the materials in complete DMEM media for 24 hrs at 37 °C without agitation. The unexposed (negative control) was polypropylene and the positive control was zinc sulfate.

Cell viability using Alamar Blue

The pure extract (200 mg/ml) and 100, 50, and 25 mg/ml dilutions (prepared using complete DMEM media) of BCP, HA and TCP were added to fibroblast cells seeded at 1 x 10^5 cells/ml in 24-multiwell plates for 24 hrs. After a 24 hrs incubation period, cell viability was tested using the Alamar Blue assay (Gibco® Invitrogen). The culture was stained with Alamar blue solution (1:10) and incubated for 4 hrs at 37 °C. After incubation, the stained culture was detected by absorbance at 570 nm using a Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). Four replicates were performed for each treatment (ISO 10993-5, 2009).

Minimum essential medium (MEM elution) study

The pure extract (200 mg/ml) and 100, 50, and 25 mg/ml dilutions (prepared using complete DMEM media) of BCP, HA and TCP were added to fibroblast cells seeded at 1 x 10^5 cells/ml in 24-multiwell plates for 24 hrs. After a 24 hrs incubation period, cells were examined microscopically under an inverted light microscope (Olympus, Tokyo, Japan) and graded based on Table 1. Four replicates were performed for each treatment (ISO 10993-5, 2009).

Cellular morphology

The pure extracts (200 mg/ml) of BCP, HA and TCP were added to fibroblast cells seeded at 1 x 10^5 cells/ml in 6-multiwell plates for 24 hrs. The plates were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. Observations of cell morphology were made on days 1, 3, 5, and 7 under an inverted light microscope (Olympus, Tokyo, Japan).

Table 1. Qualitative morphological grading of cytotoxicity of extracts

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observables
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50% growth inhibition observables
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50 % growth inhibition observables
4	Severe	Nearly complete or complete destruction of the cell layers

RESULTS AND DISCUSSION

Phase analysis

Figure 1 presented the XRD analysis results of the BCP, HA and TCP. It is clearly shown that BCP spectra has distinct peaks of HA and TCP compounds. This suggests that BCP did not change into other phases during sintering. Meanwhile, the samples of single HA and TCP exhibited only characteristic peaks of HA and TCP, respectively.

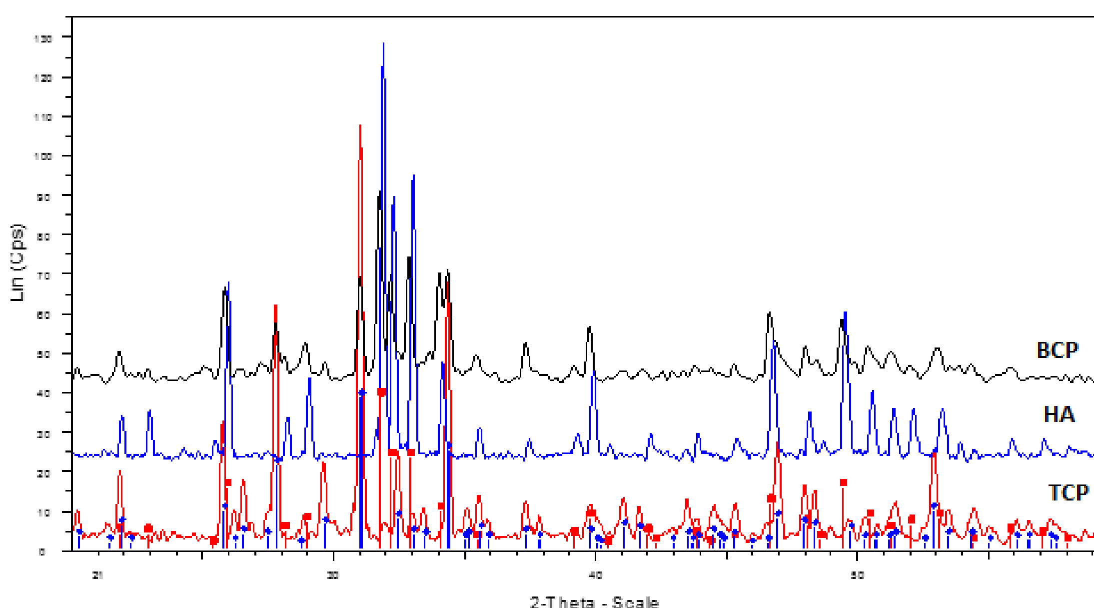

Figure 1. XRD pattern of BCP, HA and TCP

Cytocompatibility assessment of BCP, HA and TCP using Alamar Blue assay and MEM elution

L929 is a standardized and continuous (established) cell line commonly used for cytotoxicity testing (recommended by ISO 10993-5, 2009) (Maria *et al.*, 2014). Alamar Blue assay was conducted to measure cell viability. Baker *et al.* reported that 50 % of cell viable and onwards indicate that the material is non-cytotoxic, whereas less than 50 % cell viability shows that the material is cytotoxic (Baker *et al.*, 2009). As shown in Figure 2, all materials were cytocompatible. No significant difference was observed between BCP, HA and TCP in term of cell viability. The extracts induced cell viability and cell growth increment, thus showing no toxic effects. Liping *et al.* has reported that L929 cells showed high growth rate and proliferation characteristics on the CaP coated magnesium alloy (Liping *et al.*, 2009).

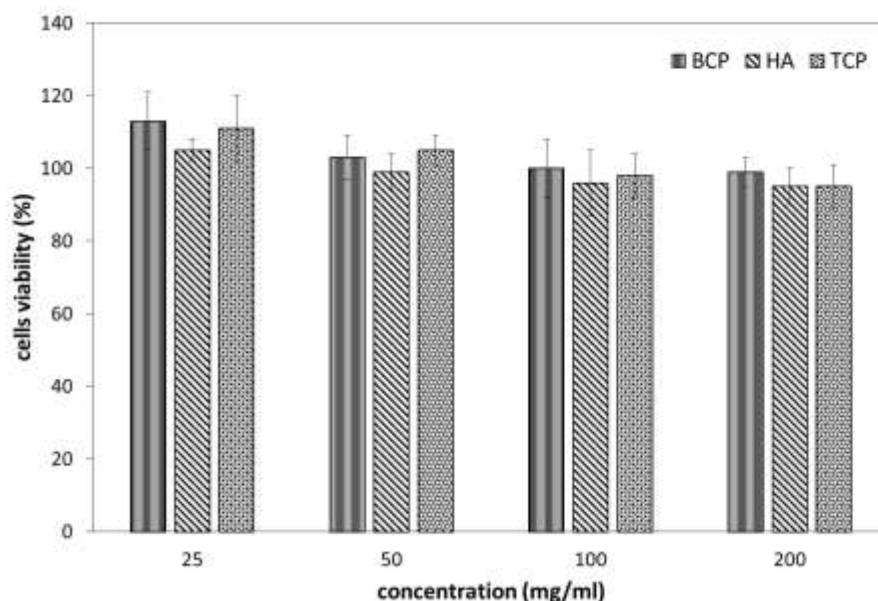

Figure 2. Cell viability of BCP, HA and TCP extracts using Alamar Blue Assay. The data are expressed as mean ± standard deviation ($p>0.05$)**.**

MEM elution study was conducted to examine morphological changes and cytolysis. Discrete intracytoplasmic granules, no cell lysis and no reduction of cell growth were observed on all cultures incubated with BCP, HA and TCP extracts. Therefore, all concentration was given a score of '0-0-0' thus exhibited 'none' reactivity based on Table 1 and comparable with negative control. However, positive control was given a score of '4-4-4' that exhibited 'severe' reactivity because all cultures condition was a complete destruction of the cell layers.

No changes in cellular morphology of BCP, HA and TCP

Cellular morphology observation conducted with an inverted light microscope exhibited no changes between cells treated with BCP, HA and TCP. On day 1 (Figure 3A) L929 cells were shown to be viable and demonstrate significantly good adherence with spherical, polygonal and elongated morphology. Some L929 cells were in the early stage of proliferation as observed in mitosis-like process. On day 3, the cells were proliferating continuously, grown and occupied the spaces between

them (Figure 3B). It is clearly shown that the cell density was increasing proportionally with incubation period (day 7 > day 5 > day 3 > day 1).

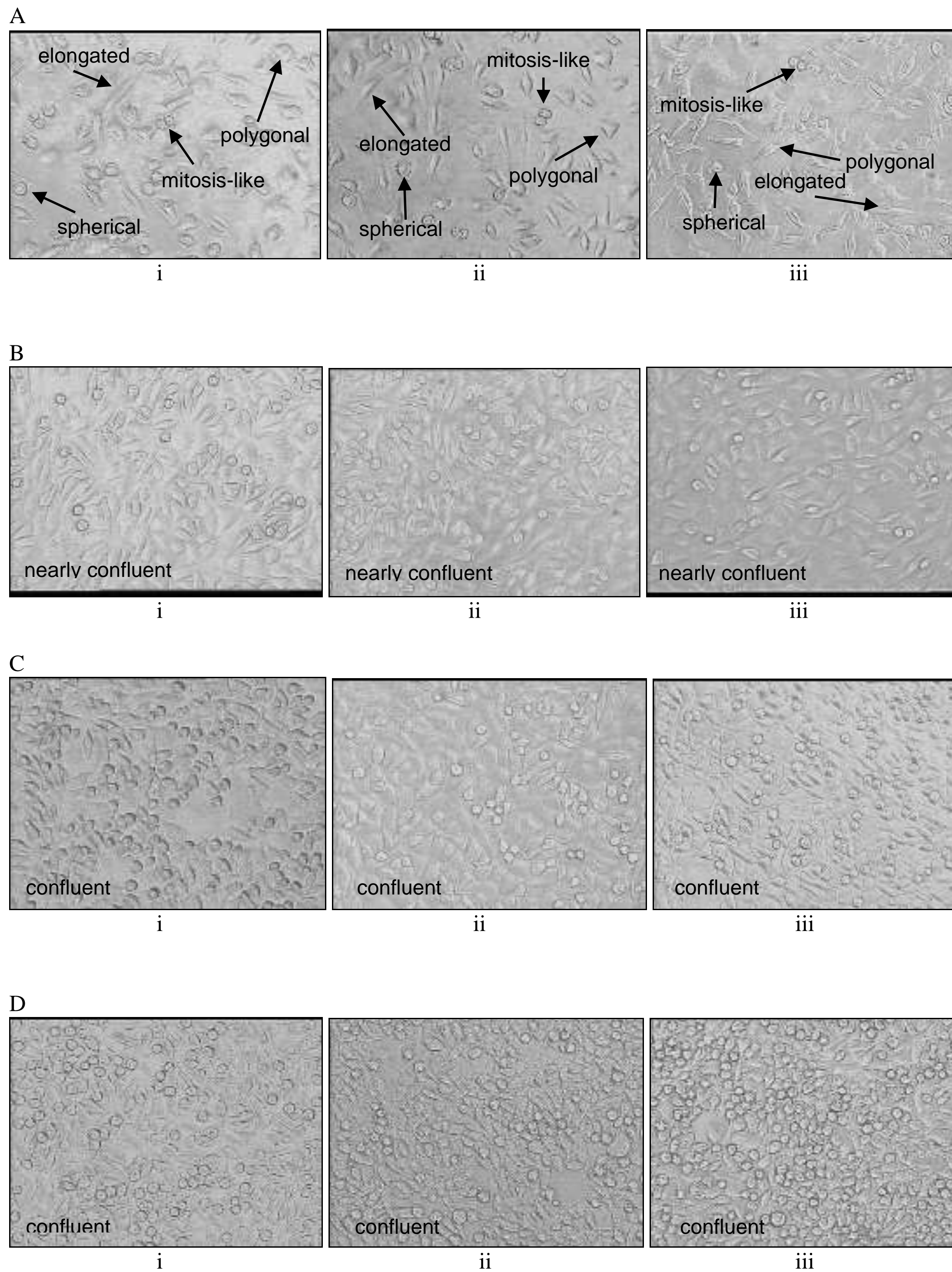

Figure 3. Micrograph (100x) of L929 cells incubated for (A) 1 day, (B) 3 days, (C) 5 days and (D) 7 days with (i) BCP, (ii) HA and (iii) TCP extract, respectively.

Interestingly, compact cell density with confluent monolayer could be observed in Figure 3C and D compared to others indicating that Figure 3D also showed that L929 cells were still viable, proliferating and no cell death observed. The important observation is that cell adhesion properties of BCP is comparable with HA and TCP. Earlier findings have demonstrated that calcium phosphate-titania composite showed good cell adhesion as well as cell-cell interaction. Cells are attached closely with neighboring cells, and form a cellular network (characteristic of fibroblast type cells) on the composite surface (Shektar *et al.*, 2009). According to previous study by Kiyoshi *et al.,* cultured L929 indicated that collagen sheet having the calcium phosphate layer on the top, supported the adhesion and growth of the cells (Kiyoshi *et al.*, 2004).

CONCLUSION

It can be concluded that BCP is safe and cytocompatible to L929 fibroblast cells up to 7 days of culture which is comparable to commercially available HA and TCP.

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