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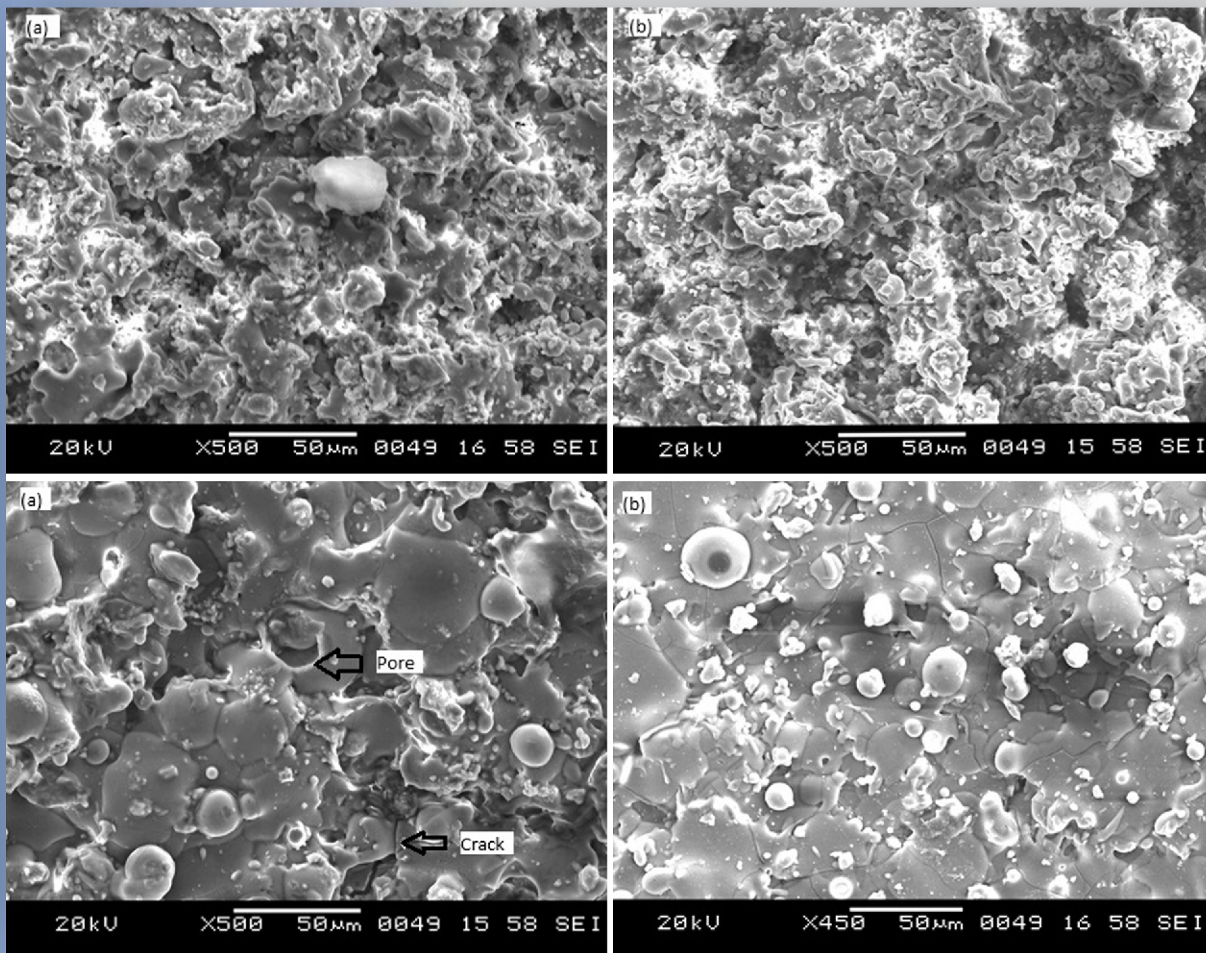

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Cytotoxic and the proliferative effect of cuttlefish bone on MC3T3-E1 osteoblast cell line

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ABSTRACT

Objective: To evaluate the cytotoxic and the proliferative effect of cuttlefish bone on MC3T3-E1 osteoblast cell line. **Materials and Methods:** MC3T3-E1 cells were treated with 0.5, 1, 5, 25, 50, 100, or 200 µg/ml cuttlefish bone powder (CBP). Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. This assay was also used to determine cell proliferation over 16 days of treatment with 0.5, 25, or 100 µg/ml CBP. **Results:** CBP was not cytotoxic to MC3T3-E1 cells at any concentration. The percentage of cell viability in the 0.5–200 µg/ml CBP groups dose dependently decreased from 107.52 ± 11.03 to 92.48 ± 5.60%; however, the differences between the groups or the negative control group were not significant. At 16 days, 0.5, 25, and 100 µg/ml CBP groups showed 123.19 ± 10.07%, 126.02 ± 15.69%, and 133.33 ± 11.74% proliferation, respectively, that were significantly higher than that of the control group. **Conclusion:** These results indicate that CBP promotes osteoblast proliferation and may be a potential material to increase the number of osteoblasts in a bone defect in the oral cavity.

Key words: Cuttlefish bone powder, cytotoxicity, osteoblast cell culture, proliferation

INTRODUCTION

Cuttlefish bone (CB) is a natural biomaterial source from the chamber of the cuttlefish that can be ground into a powder. CB is a brittle structure found in all members of the cephalopod family and is a chambered, gas-filled shell used to control floating.[1] A gas and liquid mixture osmotically regulates the pressure inside the CB.[2,3] The main chemical CB components are 87.3%–91.75% calcium carbonate and chitin. In addition, CB also contains trace amounts of silicon, aluminum, titanium, manganese, barium, and copper.[4] CB is a traditional Chinese medicine that is effectively used in treating gastritis and frequently used as a hemostatic agent after tooth extraction or rhinoplasty.[4] Moreover, the synthesis of Hydroxyapatite (HAp) from natural biomaterials, such as eggshell,[5] coral,[6] and CB[7] has been reported.

Materials that enhance bone regeneration have a wide range of potential clinical applications, from treating nonunion fractures to spinal fusion. The use of porous material scaffolds with bioceramic and polymer components to support bone cell and tissue growth is a popular research topic. Current challenges include engineering materials that can match the mechanical and biological properties of the bone tissue matrix and support the vascularization of large tissue constructs.[8] The most common biomaterials used for

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bone tissue engineering include Hap, titanium, alumina, and polymers.[9] Battistella *et al*.[10] revealed that cell proliferation in a three-dimensional (3D) CB scaffold increased. They also suggested investigating the use of dynamic culture to improve cell proliferation and differentiation. The mechanical properties of natural bone are of interest in bone tissue engineering. A scaffold should have a highly porous matrix for transporting nutrients, oxygen, and metabolic products.[11] In addition, cuttlefish bone powder (CBP) was added to paste or gel dentifrices or used directly with a toothbrush to clean the teeth and improve oral hygiene.[12,13] We would like to investigate the biological properties of CB available in Thailand. In the present study, the biocompatibility and effect of CBP on MC3T3-E1 osteoblast cell proliferation were evaluated *in vitro*.

MATERIALS AND METHODS

Cuttlefish bone powder preparation

The bone inside the cuttlefish (from the Southern part of Thailand) was removed and cut in the middle into small pieces (1 cm × 1 cm × 0.5 cm) [Figure 1]. The CB was rinsed with deionized water, then boiled 10 min for getting rid of the odor and microorganisms. To desorb any impurities, the CB was dried at 103°C–105°C for 24 h and cooled in a desiccator at room temperature. The CB was crushed, pulverized,

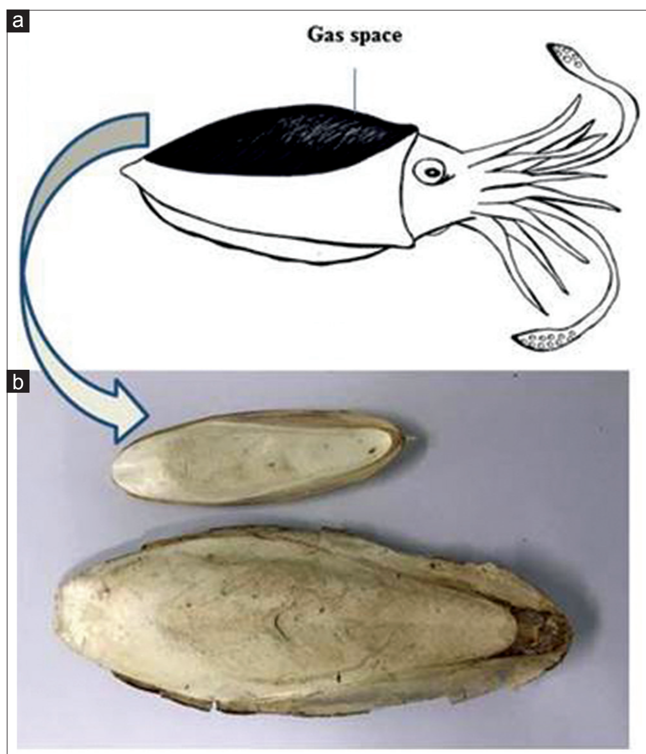

Figure 1: (a) Cuttlefish bone is the hard tissue in cuttlefish that functions in floatation. (b) Natural cuttlefish bone

and sieved (Pass 80 mesh) into a 150–250 μm particle powder. The CB powder (CBP) was used as the test material in the experiments.

Test materials

The CBP had a maximum 8% moisture and a pH range of 6.0–8.0. The minimum powder fineness passed through a No. 80 sieve with 75% efficiency for sterilization before cell culture experiment. The CBP (200 mg) was mixed with 1 ml of Dulbecco's modified Eagle's medium (DMEM, Invitrogen, CA, USA) for a 20% (w/v) solution. The solution was incubated at 37°C in a 5% CO_2 atmosphere for 24 h, per ISO 10993-12.[14] The CBP stock solution was centrifuged at 3500 rpm for 10 min, and the supernatant was diluted into 0.5, 1, 5, 25, 50, 100, or 200 μg/ml solutions.

We used 3 cm^2 polyurethane/2 ml of DMEM (Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan) as a positive control per ISO 10993-5.[15] The polyurethane films were sterilized by soaking in 70% alcohol for 1 min, washed in normal saline for 1 min, and left to dry. The dry films were immersed in DMEM and incubated at 37°C in a 5% CO_2 atmosphere for 24 h before testing.

Thermanox® Coverslips (NUNC™ Naperville, IL, USA) (6 cm2/2 ml of media) served as a negative control per ISO 10993-5.[15] Thermanox® Coverslips were cut into small pieces, soaked in DMEM, and incubated in a 5% CO_2 atmosphere at 37°C for 24 h before testing.

Cell culture procedure

The cells used in this experiment were a continuous cell line, MC3T3-E1Subclone 4 Strain C57BL/B mouse osteoblast-like cell line (ATCC® CRL-2593™, USA). The cells were maintained in DMEM containing 10% fetal calf serum, 200 μg/ml penicillin G, 200 μg/ml streptomycin, and 2 μg/ml fungizone at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed every other day. When the cells reached confluence, they were detached using 0.2% (w/v) trypsin and transferred to new culture flasks.

Cytotoxicity evaluation

At 80% confluency, the cells were trypsinized and plated in 96-well culture plates (1 × 10^4 cells/well). Each well contained 100 μl of cell suspension, and the plates were incubated for 24 h at 37°C in a 5% CO_2 atmosphere. After 24 h, the media was removed from each well. Subsequently, 100 μl of eluent from the 0.5, 1, 5, 25, 50, 100, or 200 μg/ml CBP solutions or the positive/negative control was placed into the 96-well culture plates (8 wells/test material). After incubation for 24 h at 37°C in

a 5% CO_2 atmosphere, cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The experiments were repeated in triplicate.

The mean optical density of the blank control group was set at 100% viability. The results for the experimental, positive control, and negative control groups were normalized to the blank control group. Statistical analysis was performed using the nonparametric Mann–Whitney test ($P < 0.05$). The relative cell count ratio was calculated from the following formula:

$$\%\,\text{Cell viability} = \frac{\text{O.D.}_{570e} - \text{O.D.}_{570b}}{\text{O.D.}_{570c} - \text{O.D.}_{570b}} \times 100$$

Where O.D._{570e} is the mean optical density of the 100% extracts of the test sample, O.D._{570c} is the mean optical density of the control, and O.D._{570b} is the mean optical density of the blanks.

Cell proliferation evaluation

MC3T3 cells were cultured in α-Minimum Eagle's Medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The cells were treated with 0.25% trypsin for 5 min at 37°C and diluted with α-MEM containing 10% FBS to a concentration of 1×10^5 cells/ml. The cells (2×10^3 cells/well) were seeded in seven 96-well culture plates (100 μl/well) and incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h. The cells were treated with 0.5, 25, or 100 μg/ml of CBP solution and the media control group (10 wells/concentration/duration) for 1, 3, 5, 7, 10, 14, and 16 days. The MTT assay was used to determine cell proliferation at each concentration at each time point.

The percentage of cell proliferation of the three experimental groups was calculated using the mean optical density from 7 to 10 days this means the values from 7 to 10 days and was expressed as a percentage of the control values.

$$\%\,\text{cell proliferation} = \frac{\text{rate of change (OD) experiment group from day 7 to day 10}}{\text{rate of change (OD) control group from day 7 to day 10}} \times 100$$

Statistical analysis

Statistical analysis was performed using SPSS-18.0 software (SPSS Inc., IL, USA). The results are presented as the mean ± standard deviation. Statistical analysis was performed using Student's t-test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

We determined the effect of CBP on MC3T3-E1 cell viability as percentage of cell viability [Figure 2]. CBP was not toxic to the MC3T3-E1 cells at any tested concentration. The percentage of cell viability in the 0.5–200 μg/ml CBP groups dose dependently decreased from 107.52% ± 11.03% to 92.48% ± 5.60%, however, these differences were not significantly different from each other or the negative control group ($P > 0.05$), while the positive control group showed a significant 5–6-fold reduction compared with the other groups ($P < 0.05$). The results of the cell proliferation evaluation showed that cell proliferation in the CBP groups peaked at 14 days and decreased at 16 days [Figure 3], with the 0.5, 25, and 100 μg/ml CB groups at 16 days demonstrating 123.19% ± 10.07%, 126.02% ± 15.69%, and 133.33% ± 11.74% proliferation, respectively, which was significantly higher compared with the media control [Figure 4].

DISCUSSION

Tissue engineering in dentistry is a multidisciplinary field. The purpose of tissue engineering is to repair, maintain, or enhance tissue and organ regeneration. Promoting the organization of cells in a 3D architecture directs the growth and formation of the desired tissue. Bone has a low capacity for self-repair due to its limited vascular supply and low rate of chondrocyte mitosis. Currently, osteoconductive porous biodegradable materials are used in tissue engineering for bone

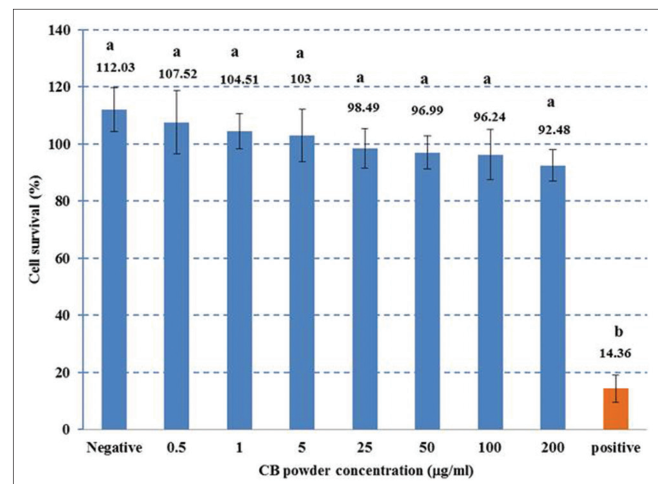

Figure 2: Cell viability percentages in the CBP, negative control, and positive control groups. Different superscript letters signify a significant difference between groups ($P < 0.05$)

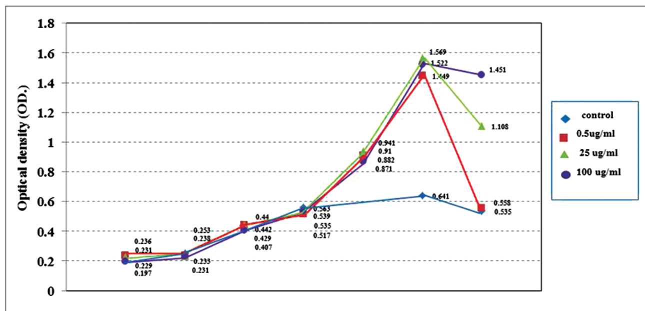

Figure 3: Cell proliferation as demonstrated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide optical density results from 1–16 days

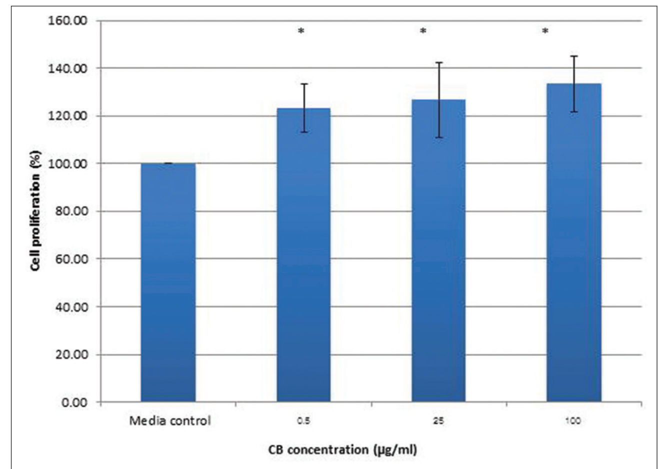

Figure 4: Percentage of cell proliferation in the 0.5, 25, and 100 μg/ml cuttlefish bone groups at 16 days. *Indicates a significant difference between the control group ($P < 0.05$)

repair. Once the bone healing is accomplished, the newly formed tissue undergoes physiologic bone remodeling, which involves the coordinated action of osteoblasts and osteoclasts.[16]

Calcium phosphate-based materials can be used as a biomaterial for tissue engineering. Hap, $Ca_{10}(PO_4)_6(OH)_2$, is widely used as a bone substitute.[17] The composition of Hap is the same as the mineral constituents in hard tissue, i.e., bone and teeth. Hap has several beneficial properties; it is nontoxic, osteoconductive, and biocompatible. Natural Hap biomaterials, such as coral and eggshell, have been recommended as the materials of choice in bone tissue engineering.[5] CB is a Hap material that has been used in dentistry for bone repair. There are two benefits of CB Hap in bone tissue engineering: the main component of CB is aragonite ($CaCO_3$) that has been converted into Hap and it also has a porous structure and bone-like architecture.[18]

Recently, Hap with a porous morphology has been used as a scaffold. The scaffold is placed in the bone defect area. This porous scaffold is beneficial for bone defect repair due to its effectiveness in cell attachment, differentiation, and proliferation, generating bone healing.[19] Polycaprolactone is a polymer commonly added to a Hap scaffold to increase its mechanical properties.[20]

CB has been proposed as a suitable material for a bone tissue scaffold. We selected MC3T3-E1 as the target cells in our experiment because they are cell line and have the capacity to differentiate into osteoblasts. MC3T3-E1 has been established from a C57BL/6 mouse calvaria and selected on the basis of high alkaline phosphatase activity in the resting state. Our study revealed the same biocompatibility with MC3T3-E1 osteoblast cell line as shown in other studies.[10,18] None of the CBP concentrations we evaluated were cytotoxic to the MC3T3-E1 cell line. The percentage of cell viability was rather high and similar to that of the negative control, while that of the positive control group was significantly fold lower.

We evaluated cell proliferation using three levels of CBP concentration; low (0.5 μg/ml), middle (25 μg/ml), and high (100 μg/ml) along with media controls. We found that the exponential phase of cells treated under these conditions was between 7 and 10 days. The percentage of cell proliferation was calculated from the optical density in the exponential phase of the CB and control groups. The low-, medium-, and high-CB groups demonstrated percent cell proliferation of 123.17%, 124.02%, and 133.33%, respectively, and were significantly higher from the media control group at 16 days. Our results correspond with those of Kim *et al.*,[21] where PCL/CB-Hap scaffold implantation generated significantly higher new bone formation. Yildirim *et al*.[22] found that the mineral composition of CB was compatible with human bone tissue and suggested its use as a scaffold. In addition, Kannan *et al*.[23] estimated that a CB channel size of 100 × 200 μm would be beneficial for bone ingrowth. Moreover, CB and shrimp shell-derived chitosan displayed good biocompatibility and supported cell attachment and growth.[24]

A study reported that raw CB contains 0.05 ppm mercury, 0.52 ppm copper, 2.42 ppm zinc, 0.39 ppm lead, and 0.07 ppm cadmium and is not cytotoxic *in vitro*.[25] Zreiqat *et al*.[26] suggested that implant surfaces coated with Mg^{2+} promote optimal osteogenesis and lead to the maintenance of nature and healthy bone.

The Mg^{2+} ion has an important role in integrins binding to their respective ligands. Integrins transduce signals from the extracellular environment to the interior of the cell or vice versa for cellular migration, adhesion, proliferation, and differentiation.[27] The Mg^{2+} present in CB is a likely reason for the cell proliferation observed in our study.

CONCLUSION

The current study revealed that 0.5–200 μg/ml CBP were biocompatible with the MC3T3-E1 cell line and that 0.5–100 μg/ml CBP induced a high percentage of cell proliferation compared with control. These findings suggest that CB has the potential to improve *in vivo* bone defect healing by increasing cell proliferation.

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Conflicts of interest
There are no conflicts of interest.

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