ORIGINAL RESEARCH

The Effect of Cuttlefish Bone on Cell Migration and Mineralization Using Boyden Chamber, Alizarin Red S, and Alkaline Phosphatase Assays

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Abstract

Aims: The effectiveness of cuttlefish bone powder (CBP) in inducing mineralization and cell migration of the osteoblast cell line (MC3T3-E1) was investigated.

Materials and methods: The cytotoxicity of 0.5, 1, 5, 25, 50, 100, or 200 µg/mL CBP on an osteoblast cell line was evaluated using the 3-(4,5-dimethylthiazol-2-yl)—2,5-diphenyltetrazolium bromide (MTT) assay. A Boyden chamber assay was used to evaluate the migration of osteoblasts treated with 0.5, 25, or 100 µg/mL CBP. The mineral deposition of treated cells with CBP in the osteogenic medium was determined on days 7, 14, and 28 using alizarin red staining. The area of the calcified nodules was determined on day 28. Analysis of variance and the Mann–Whitney *U* test were used to compare the difference between the groups. The alkaline phosphatase (ALP) activity of the target cell treated with 0.5 µg/mL CBP was analyzed by using the ALP colorimetric assay kit.

Results: All CBP test concentrations were nontoxic to the osteoblast cells. Cell migration after 16 hours in the 0.5, 25, and 100 µg/mL CBP group was 127, 112, and 113%, respectively, compared with the control. The calcified nodule formation on day 28 was greater than on days 7 and 14 and in control groups. ALP expression was found to increase significantly after 28 days of treatment with CBP.

Conclusion: The results demonstrate that CBP induces mineralization and cell migration of the osteoblast cell line and the expression of the ALP enzyme.

Clinical significance: The cuttlefish bone is a biomaterial that not only induces cell migration but also mineral deposition. The scaffold made from cuttlefish should be suitable for clinical use for bone regeneration.

Keywords: Cuttlefish bone, Migration, Osteogenic differentiation.

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Introduction

Bone regeneration is a process of bone healing after an injury where the defect area is restored with the original tissue type rather than scar tissue. However, when the bone defect becomes larger, bone tissue regeneration is induced using bone autografts (the optimum method), allografts, and biocompatible biomaterials that are colonized by host cells that migrate from the surrounding tissue and differentiate into osteoblasts.[1]

Guided tissue regeneration (GTR) occurs following the application of biocompatible biomaterials with different compositions and variable porosity around the periodontal defect. Bone can grow into a defect if space is provided. The GTR membrane acts as a barrier to prevent fast-growing gingival tissue cells from populating the root surface and maintains space for new bone regeneration. When treating bone defects or alveolar ridge augmentation, the GTR membrane should prevent the epithelium and connective tissue from migrating into the defect while allowing the migration and differentiation of osteoblasts. The use of a natural biological membrane or scaffold, such as seashells, animal bones, coral, and cuttlefish bone (CB), for tissue engineering has been investigated due to the limitations of synthetic materials with the desired structure and mechanical integrity.[2–4] Porous scaffolds have been widely accepted as an appropriate material for transporting nutrients, oxygen, and metabolic products into the defect area to achieve bone tissue growth and vascularization.[5] CB is mainly composed of aragonite and can be transformed using hydrothermal processing to hydroxyapatite (Hap) and is one of the recommended natural Hap materials for bone repair.[6] CB-based scaffolds for bone regeneration are suitable for clinical use in implant or tissue-engineering applications because they have demonstrated excellent bioactivity *in vitro*. The experiment of Rocha et al. revealed that a distinct formation of Hap appeared when the CB scaffold was immersed in simulated body fluid, and remarkable osteoblast proliferation was observed.[4] Kim et al.[7] also demonstrated that CB increased the adhesion, proliferation, and differentiation of human mesenchymal stem cells *in vitro*. They also

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suggested CB could be used as a bone substitute as a barrier to prevent fibroblasts from migrating into the bone defect during bone regeneration. Moreover, a scaffold should have proliferative and chemotactic effects to recruit osteoblasts to migrate into the wound area for bone healing. Analysis of the cuttlebone of the cuttlefish *Sepia officinalis* revealed that the CB develops from successive calcium carbonate lamellae being formed during growth. The rhythm of lamellae deposition is not constant and has been shown to be closely linked with temperature.[8] Choe[9] also confirmed that the rhythm of formation is constant at temperatures between 19 and 30°C in *Sepia esculenta* and *Sepia subaculeata*. Moreover, both the growth rate of the body mantle and temperature influence the elemental ratio of lithium/calcium and strontium/calcium in CB.[10] Therefore, there are variations in CB from different environments. The bioactivity of the CB available in Thailand on cell migration and its osteoinductivity has not yet been reported. The purpose of this study was to evaluate the effects of cuttlefish bone powder (CPB) on the migration and osteogenic differentiation of MC3T3 osteoblast-like cells.

Materials and Methods

Cuttlefish Bone Powder (CBP) Preparation

The bone inside the cuttlefish, *Sepia officinalis*, from the Southern part of Thailand (Twin Lotus Co Ltd., Bangkok, Thailand) was removed and cut into 1 × 1 × 0.5 cm pieces. The CB was rinsed with deionized water and boiled in a pot with a lid for 10 minutes to remove any odor or microorganisms. The impurities were desorbed by drying at 103–105°C for a day and cooled in a desiccator at room temperature. The CB was crushed, pulverized, and sieved (Pass 80 mesh) into a 150–250 µm particle size.

Test Materials

The CBP had a maximum of 8% moisture and a pH range of 6.0–8.0.[11] The minimum powder fineness passed through a No. 80 sieve with 75% efficiency for sterilization before using it in the cell culture experiments. The CBP (200 mg) was mixed with 1 mL of Dulbecco's modified Eagle's medium (DMEM) (DMEM, Invitrogen, California, United States of America) for a 20% (w/v) solution. The solution was incubated at 37°C in a 5% CO_2 atmosphere for 24 hours, per ISO 10993-12.[12] The CBP stock solution was centrifuged at 3500 rpm for 10 minutes, and the supernatant was diluted into 0.5, 25, and 100 µg/mL solutions. Polyvinyl chloride (PVC) (PVC; Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan) and Thermanox plastic coverslips (NUNC™, Naperville, Illinois, the United States of America) are used as a positive and negative control, respectively.[13]

Cell Culture Procedure

The cells used in this experiment were a continuous cell line, the MC3T3-E1subclone 4 strain C57BL/B mouse osteoblast-like cell line (ATCC® CRL-2593™, the United States of America). 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. Cells were transferred to new culture flasks after reaching confluency.

Cytotoxicity Evaluation

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 hours. After the incubation period, the media was removed from each well. Subsequently, 100 µL 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 (eight wells per test material). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)—2,5-diphenyltetrazolium bromide (MTT) assay after a 24-hour incubation period. The experiments were repeated three times.

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 *U* test ($p < 0.05$). The relative cell count ratio was calculated from the following formula:

$$\text{Cell viability (\%)} = [(OD_{570e} - OD_{570b})/(OD_{570c} - OD_{570b})] \times 100$$

Where optical density (OD_{570e}) is the mean optical density of the 100% extracts of the test sample, OD_{570c} is the mean optical density of the control, and OD_{570b} is the mean optical density of the blanks.

Boyden Chamber Assay

The cells were trypsinized and plated in the top of the transwell inserts in a 24-well transwell culture plate at a concentration of 2.5×10^5 cells/well/200 µL serum-free medium. The lower chamber of the transwell culture plate contained 750 µL of culture medium and 5% fetal bovine serum with 0.5, 25, or 100 µg/mL CBP (the three levels of concentrations of CBP were randomly selected for cell migration). CBP was not added in the media for the control group wells. The experiment was performed using three wells per group, and the plates were incubated at 37°C in a 5% CO_2 atmosphere. After 16 hours, the media were removed from the wells. The cells were fixed with 100% methanol at room temperature for 10 minutes and stained with 10% Giemsa stain for 1 hour at room temperature. After 1 hour, the Giemsa stain was removed by rinsing it with distilled water. The nonmigrating cells on the top of the transwell were wiped off with a cotton bud. The migrating cells were counted using the Image J Program Version 2.0. Each experiment was performed three times.

Osteogenic Differentiation Assay

Osteoblast cells, MC3T3-E1 cells (1×10^5 cells/well), were seeded in a 6-well plate (Costar, Corning) and cultured until approximately 90% confluent. The cells were then treated with an osteogenic medium (MEM-α containing 10 mM β-glycerophosphate, 10 nM dexamethasone, and 50 mg/mL ascorbic acid).[14] The osteogenic medium was changed every other day during osteogenic differentiation for 28 days. In the three experimental groups, the osteogenic medium was supplemented with 0.5, 25, or 100 µg/mL CBP and was added into three wells (one well/concentration). The osteogenic medium and 10% MEM-α were added in separate wells as negative and positive control wells, respectively. The media was changed every other day to maintain a constant concentration of CBP in the experiment groups. The experiment was repeated three times. On days 7, 14, and 28 of culture in osteogenic medium, calcium deposition was analyzed from the 0.5, 25, and 100 µg/mL CBP, negative, and positive control groups by fixing the cells in 10% formalin and staining with 1% alizarin red S. Mineralized matrix nodule formation was determined by this staining. Briefly, the cells were fixed in 95% ethanol for 30 minutes at room temperature. After washing with PBS, alizarin red (pH = 4.2) was added and incubated for 30 minutes at room temperature. Images of the staining on days 7, 14, and 28 were recorded, and quantitative analysis was performed on day 28 using Amersham™ ImageQuant™ 800 (Cytiva, Marlborough, Massachusetts, the United States of America).

Alkaline Phosphatase (ALP) Activity Analysis

The MC3T3-E1 cells (1×10^5 cells/well) were seeded in 24-well plates (three plates per cycle). The experiment was repeated in triplicate. In each plate, three wells per condition (positive control, negative control, and sample group) was designed. The cells were treated with 100 µL of 0.5 µg/mL of CBP (the lowest concentration was selected). The positive control was osteogenic medium (MEM-α containing 10 mM β-glycerophosphate, 10 nM dexamethasone, and 50 mg/mL ascorbic acid). The negative control was 10% MEM-α. The media was changed every other day until days 7, 14, and 28. The cell was lysed by lysis buffer. The cell suspension was put in a 1.5 mL Eppendorf tube and kept at –85°C for ALP activities measurement. ALP activities of the cell were detected with ALP colorimetric Assay Kit (Abcam® Cambridge, the United Kingdom). ALP enzyme in the supernatant catalyzed the transphosphorylation reaction of p-nitrophenyl phosphate to p-nitrophenol, and the clear solution changed to yellow color.

Statistical Analysis

The cell viability data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) 18.0 software for Windows (SPSS Inc, Chicago, Illinois, the United States of America). To analyze the data, the mean of the data collected from the seven concentrations and negative and positive controls were analyzed using one-way analysis of variance (ANOVA). The difference between the control group and the test group was determined by the Mann–Whitney *U* test for independent samples. Differences with $p < 0.05$ were considered statistically significant. The CBP samples of percentage cell migration and the osteogenic differentiation of cells were measured and statistically compared by ANOVA and the Mann–Whitney *U* test. Statistical significance was defined as $p < 0.05$. The CBP samples of ALP activity analysis were measured and statistically compared by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test. Statistical significance was defined as $p < 0.05$.

Results

Cell Viability

The cytotoxic effect of the CBP on MC3T3-E1 cell viability is presented as percentage cell viability (Fig. 1). The percentage cell viability in the 0.5–200 µg/mL CBP groups dose-dependently decreased from 107.52 ± 11.03–92.48 ± 5.60%. These results indicated that 0.5–200 µg/mL CBP was not cytotoxic to the MC3T3-E1 cells. Thus, 0.5, 25, and 100 µg/mL CBP were used in the subsequent experiments evaluating cell migration.

Cell Migration

In the Boyden chamber assay, at 16 hours, the mean of cell migration of the control group was 1,855.67 cells, equal to 100% cell migration, while cell migration of 0.5, 25, and 100 µg/mL CBP groups were 2,357; 2,078; and 2,098 cells, which is equal to 127.50 ± 15.63%, 112.63 ± 11.55%, and 113.07 ± 11.96%, respectively compared with the control group (Fig. 2) and were significantly higher than that of the control group.

Osteogenic Differentiation

The mineralization effect of CBP on MC3T3-E1 osteoblasts was determined using alizarin red staining (Fig. 3). The 0.5, 25, and 100 µg/mL CBP groups demonstrated nodule areas of 267,245 ± 799 pixels/well, 271,950 ± 2,129 pixels/well, and 276,782 ± 3,529 pixels/well, respectively (Fig. 4 and Table 1).

Alkaline Phosphatase (ALP) Activity Analysis

The results of intracellular ALP measurement are reported in Figure 5. Cells treated with 0.5 µg/mL CBP at days 7, 14, and 28 show increasing ALP expression and are significantly different from the control groups ($p < 0.05$). This experiment revealed that CBP has no cytotoxicity and also induces cell migration and mineralization of the osteoblast cell line.

Discussion

The present study evaluated the effects of CBP on osteoblast-like cell cytotoxicity, migration, and osteogenic induction. The results indicated that CBP was not cytotoxic and significantly increased cell migration and mineralization of the osteoblast-like cells compared with the control.

The results revealed that CBP was nontoxic to the osteoblast cell line (MC3T3-E1) at all tested concentrations. The percentage cell viability ranged from 107.52 to 92.48% and was not significantly different from the negative control (112%, $p > 0.05$); however, these values were significantly different from the positive control

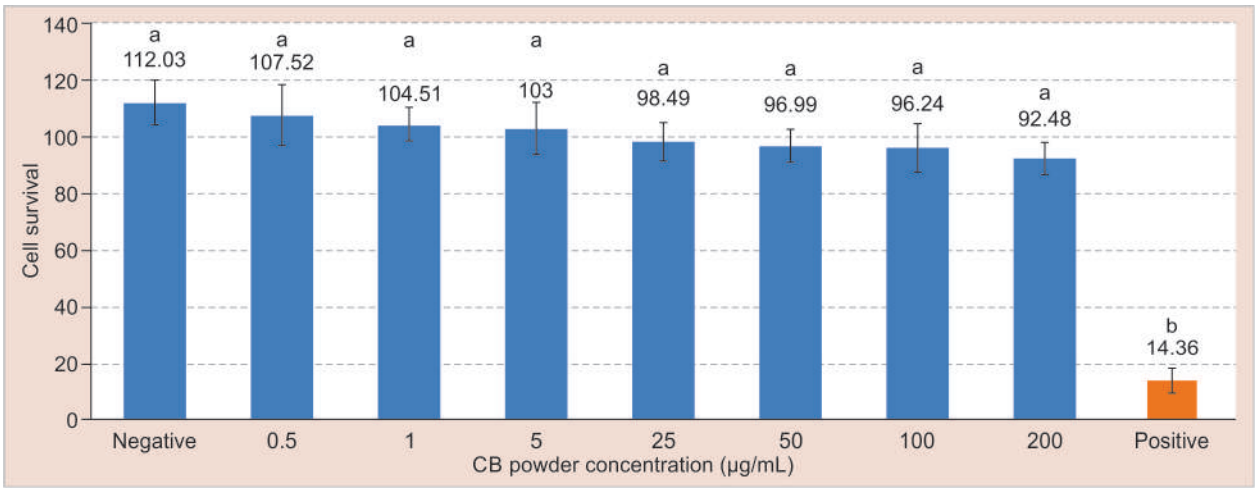

Fig. 1: Cell viability percentages in the CBP, negative control, and positive control groups. Different letters signify a significant difference between groups ($p < 0.05$)

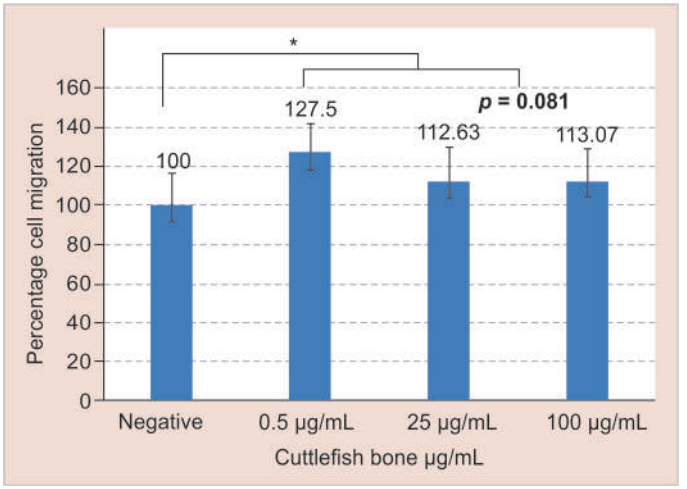

Fig. 2: Cell migration after exposure to the negative control and different concentrations of CBP *$p < 0.05$ compared with the negative control

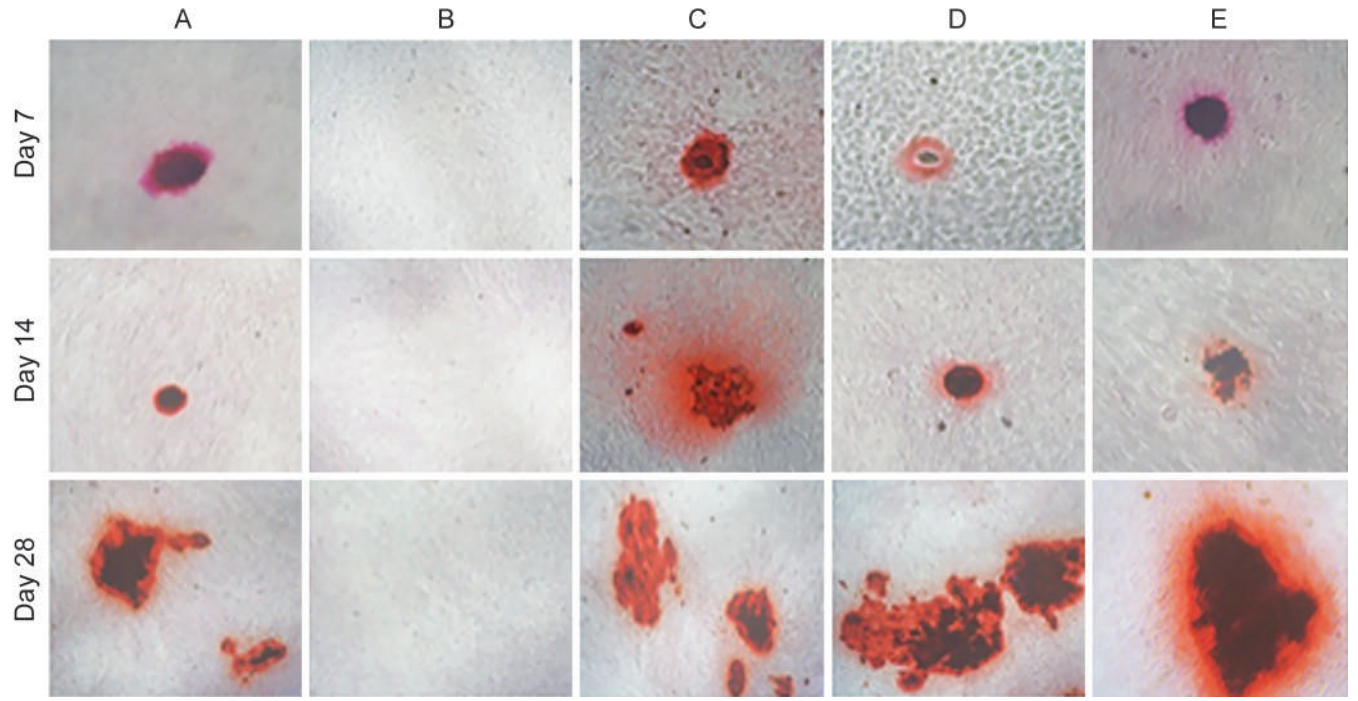

Fig. 3: Alizarin red staining of the nodules after culturing the osteoblast cell line in an osteogenic medium supplemented with three concentrations of CBP at three-time points [A, control (+ve); B, control (−ve); C, concentration 0.5 µg/mL; D, concentration 25 µg/mL; E, concentration 100 µg/mL]

(14%, $p < 0.05$). These results indicate that all tested concentrations of CBP were biocompatible with osteoblasts and should have no adverse effects *in vivo*. Results correspond with those of Vajrabhaya et al.'s study.[15] Inducing cell migration is a desired property in a biomaterial used in guided bone regeneration. Cell migration assay demonstrated a percentage cell migration of 127, 112, and 113% in the 0.5, 25, and 100 µg/mL CBP groups, respectively, compared with the control group. The CBP media induced more osteoblasts to migrate from the upper chamber of the transwell to the underside of the membrane compared with the control media ($p < 0.05$). Media containing a potential chemoattractant or therapeutic agent in the lower chamber should induce cell migration through the membrane pores. In addition, the biomaterial often has specific protein binding sites and biochemical signals that can accelerate wound healing and integration with that organ.[16] A previous study by Vajrabhaya et al. using MC3T3-E1 cells revealed 123, 124 and 133% cell proliferation when cultured with 0.5 µg/mL, 25 ug/mL, and 100 µg/mL CBP, respectively, which were significantly higher compared with the media control group at 16 days.[15] The experiment also showed by the 3-(4,5-dimethylthiazol-2-yl)—2,5 diphenyl tetrazolium bromide optical density that no cell proliferation at 14 and 3 days of the incubation period. These results indicate that the penetration of cells through the Boyden chamber membrane in the present study after 16 hours of cell incubation was due to cell migration and not cell proliferation. MC3T3 is an osteoblast precursor cell line derived from *Mus musculus* (mouse) calvaria.[17] Bilezikian et al. stated that the MC3T3-E1 sub-line is one of the most convenient and physiologically relevant systems for investigating gene expression in calvarial osteoblasts.[18] These cells are the most prevalent *in vitro* model of bone matrix mineralization. Despite their widespread use, there has been no systemic characterization of the mineral produced by these cells, and the mechanisms of extracellular matrix mineralization remain unclear.[19] The mineralized deposits from

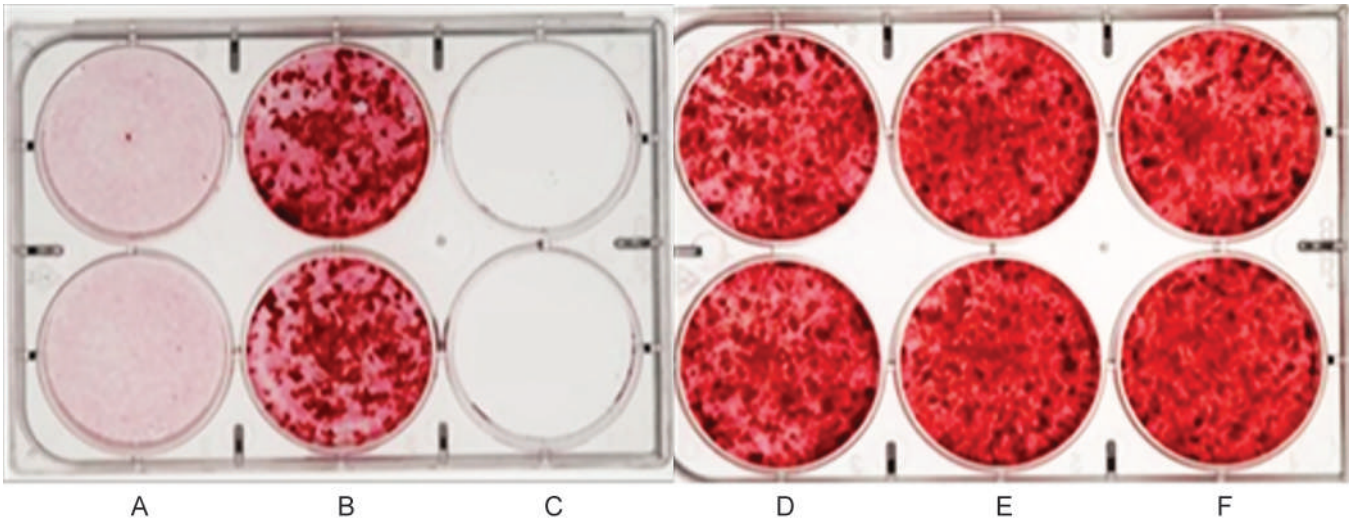

Fig. 4: Alizarin red staining of the nodules after culturing the osteoblast cell line in osteogenic medium supplemented with the three concentrations of CBP for 28 days [A, MEM-α + 10% FBS control (–ve); B, osteogenic differentiation containing medium control (+ve); C, blank wells; D, CBP 0.5 µg/mL; E, CBP 25 µg/mL; F, CBP 100 µg/mL]; each concentration was done in duplication

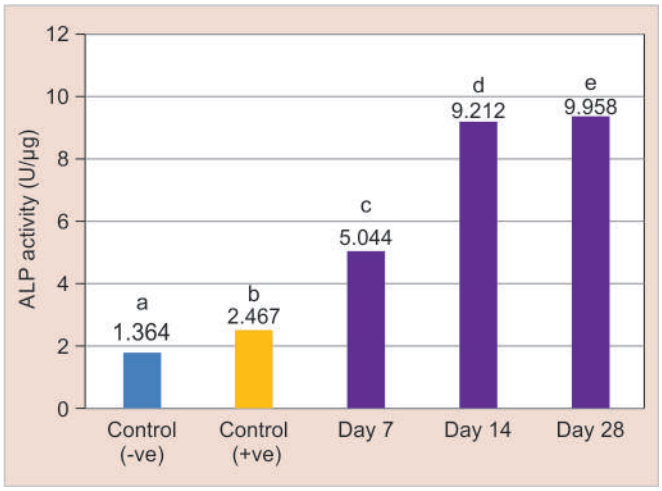

Fig. 5: ALP activity (unit/µg) of MC3T3-E1 osteoblast cells in the environment of cuttlefish bone powder media concentration 0.5 µg/mL at various days

Table 1: Nodule formation area on day 28 after culture with the MC3T3-E1 osteoblast cell line and osteogenic medium supplemented with three concentrations of CBP

Group	*Nodule formation pixel/area on day 28 (mean ± standard deviation)*
Negative control	0
Positive control	255,870 ± 2,181[a]
CBP 0.5 µg/mL	267,245 ± 799 [b]
CBP 25 µg/mL	271,950 ± 2,129 [c]
CBP 100 µg/mL	276,782 ± 3,529 [d]

The different letters indicate a significant difference ($p < 0.05$)

osteoblasts in cell culture are used as an *in vitro* model for evaluating bone cell differentiation and bone formation. The study of Langenbach and Handschel confirmed that alizarin red is a stain that is specific for calcium deposition *in vitro* bone formation.[20] Also, this study confirmed that MC3T3-E1 cells have the capacity to differentiate into osteoblasts. Rogina et al.[21] demonstrated the biocompatibility of the CB scaffolds on human embryonic kidney cells with an emphasis on cell proliferation during three days of culture.

The present study used the Boyden chamber assay for evaluating cell migration instead of the conventional scratch assay. The two assays can be performed in tissue culture plates. The scratch area produced by the pipette tip should be of a similar size in both control and experimental groups, or the discrepancies in the width of the wound area may cause variation in the results. In contrast, the Boyden chamber assay apparatus is commercially available, where membranes with the same pore size for cell migration are used in every evaluated group.[22] Furthermore, this assay is easier to perform in a cell culture laboratory and avoids some limitations in the scratch assay procedure.[23] However, both techniques generate similar results when evaluating cell migration. Evaluation of the extracellular matrix after culturing MC3T3-E1 with different treatments in osteogenic medium demonstrated increased mineral accumulation, where the higher concentration of CBP generated larger calcified nodules compared with the lower concentration and the positive control (Table 1 and Fig. 3). The high concentration of 100 mg/mL CBP exhibited more staining and nodule formation area compared with the other experimental groups at the final observation day (day 28, $p < 0.05$). ALP expression was used to quantify osteoblastic differentiation of MC3T3 E-1 cells after being treated with 0.5 µg/mL CBP for 7, 14, and 28 days. The results demonstrated the same trend as the biomineralization assay using alizarin red staining.[24] Either increasing CBP concentration or the length of incubation period extension affects the mineralization of target cells. Finally, ALP activity measurement in this study confirmed that naturally Hap transformed from CB is effective in promoting both cell migration and osteogenic differentiation and agreeable with the study of Cozza et al.[25] The limitation of the present study is the preparation of CB for testing not simulating the clinical practice. Further assessment of cuttlefish *Sepia officinalis* in membrane structure for biocompatibility evaluation should be performed.

Conclusion

This study demonstrated that CBP from cuttlefish in Thailand promotes cell migration and induces the osteogenic differentiation of MC3T3-E1 cells *in vitro*. A scaffold containing this material could

be used for guided bone regeneration by attracting cells with osteogenic potential into the defect area and regenerating bone before the more rapidly proliferating cells in the overlying soft tissues penetrate the defect.

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