รายงานโครงการวิจัยฉบับสมบูรณ์

โครงการ ผลของเปลือกหอยส่วนที่ละลายน้ำได้ต่อเซลล์สร้างกระดูกในสภาวะที่มีน้ำตาลสูง
(Effects of nacre water-soluble matrix on osteoblasts in the elevated extracellular glucose condition)

โดย ผู้ช่วยศาสตราจารย์ทันตแพทย์หญิง ดร. สุชยา ดำรงค์ศรี และคณะ

ธันวาคม ๒๕๕๑

สัญญาเลขที่ MRG4980025

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สัญญาเลขที่ MRG4980025

โครงการ: ผลของเปลือกหอยส่วนที่ละลายน้ำได้ต่อเซลล์สร้างกระดูกในสภาวะที่มีน้ำตาลสูง

Executive Summary

ชื่อโครงการ : ผลของเปลือกหอยส่วนที่ละลายน้ำได้ต่อเซลล์สร้างกระดูกในสภาวะที่มีน้ำตาลสูง
Effects of nacre water-soluble matrix on osteoblasts in the elevated extracellular glucose condition.

ระยะเวลาโครงการ: ๑ กรกฎาคม ๒๕๔๙ ถึง ๓๑ ธันวาคม ๒๕๕๑

ชื่อหัวหน้าโครงการวิจัยผู้รับทุน : ผศ.ทพญ.ดร. สุชยา ดำรงค์ศรี

ชื่อนักวิจัยที่ปรึกษา : ผศ.ดร.ปานจิตต์ ชุณหบัณฑิต

Nacre is a calcified structure that forms the lustrous inner layer of some molluskan shells. Its organic matrix has been classified as "water-soluble matrix" (WSM) and "water-insoluble matrix", based on its solubility in aqueous solutions. The WSM of nacre is believed to be intimately involved in guiding crystal nucleation and growth through molecular interactions between matrix macromolecules and mineral. Several *in vitro* and *in vivo* studies showed that the nacre is biocompatible, biodegradable, and osteoconductive. When nacre is implanted in bone, new bone formation occurs without any intervening fibrous tissue. In diabetic bone, the osteoblastic bone deposition is decreased whereas the osteoclastic bone resorption is increased resulting in delay matrix mineralization. However, very little is known about the effect of nacre WSM on osteoblast in diabetic condition.

Materials and Methods: This project was divided into three parts:

Part I To evaluate whether the water-soluble from the nacre of *Pinctada maxima* enhance the *in vitro* osteoblastic mineralization in the diabetic condition using an osteoblastic cell line (MC3T3-E1).

1.1 Extraction and purification of nacre WSM using a planetary mill (with heat generation during grinding)

1.2. Determination of the suitable concentration of WSM

1.3 Cell cultures of diabetic condition in MC3T3-E1 for markers and mineralization

1.4 Quantification of mineralized nodules using Alizarin Red S staining (only one 35 mm culture dish per group)

1.5 Analysis of bone matrix markers using real-time PCR (only one 35 mm culture dish per group)

Part II To evaluate whether the WSM or EDTA extracts enhance the matrix mineralization under the diabetic condition using human primary bone cells

2.1 New extraction and purification of WSM using a pestle and mortar

2.2 Extract and purify the EDTA-soluble matrix of nacre

2.3 Cell cultures of primary bone cells using either WSM or EDTA-soluble extract and compare the mineralization rate.

Part III To repeat Part I but using new nacre extraction technique and more culture dishes for analysis of bone markers

3.1 Extraction and purification of nacre WSM using a pestle and mortar

3.2. Cell cultures of diabetic condition in MC3T3-E1 for markers and mineralization

3.3 Quantification of mineralized nodules using Alizarin Red S staining (using two to five 35 mm culture dishes per group)

3.4 Analysis of bone matrix markers using real-time PCR (using three 35 mm culture dishes per group)

Results:

Part I 1. The suitable concentration of nacre WSM extract was 135 µg/ml

2. At 2 and 3 weeks, MC3T3-E1 cell cultures in a medium containing 50 µg/ml ascorbic acid (AA), 2 mM β-glycerophosphate (βGP) and intermittent high glucose produced mineralized nodules less than those groups without intermittent high glucose.

3. At 3 and 4 weeks, MC3T3-E1 cell cultures in a medium containing WSM produced nodules similar amounts to those groups without WSM.

4. Bone markers by real-time PCR were not suitable because no standard deviation from different culture dishes of the same groups.

Part II 1. Primary human bone cells were migrated from bone explants at day 7.

2. At 2 week, the groups with extracellular high glucose produced small sizes of nodules than those without glucose.

3. At 2 week, the culture with the presence of WSM produced slightly larger nodules that those with EDTA extract.

4. With the presence of EDTA extract, the nodule formation did not differ from the positive control (BC +AA + βGP).

5. Due to no significant amounts of mineralized nodules, we did not analyze bone matrix markers.

Part III 1. At day 16 under normal glucose level, the culture with the presence of WSM produced mineralized nodules significantly higher than that without WSM indicating the nacre WSM enhanced initial stage of mineralization.

2. At day 16, the groups with extracellular glucose produced no mineralized nodules indicating the glucose delayed initial stages of mineralization.

3. At day 29 under intermittent high glucose, the mineralized nodules were formed significantly higher than that group without glucose.

4. The cbfa1 expression of cultures with intermittent high glucose condition was increased about three times higher than those groups without glucose at day 16.

5. The BSP expression of cultures with intermittent high glucose condition was dramatically increased about five times at day 16, compared to those groups without glucose. Moreover, this expression was approximately 10 times higher than those without glucose at day 22.

Discussion:

MC3T3-E1 cell is a well-characterized preosteoblastic cell line derived from mouse calvaria. In the presence of 50 μg/ml ascorbic acid and 2 mM inorganic phosphate, this osteoblastic cell line started matrix mineralization at day 16 and the numbers of nodules were increased according to time. In the present study, human primary bone cells could not produce significant numbers of mineralized nodules.

Several studies suggested that diabetes has an effect on bone metabolism leading to osteopenia. Diabetic mice showed diminished expression of osteocalcin, Cbfa1, and collagen type I. *In vitro* studies in the presence of elevated extracellular glucose levels simulating diabetes resulted in a decrease calcium uptake and down regulation of osteocalcin at day 30 of MC3T3-E1 cell culture, a decrease in bone marrow stromal cells proliferation, alkaline phosphatase activity and the number of mineralized nodules formed, a decrease in periodontal cell proliferation and differentiation. Our results clearly demonstrate that under the intermittent high glucose condition, MC3T3-E1 cells had delayed initial mineralization but the cbfa1 expression was increased, however the expression of Col1a2 and osteocalcin at day 16 was not significantly different from those of controls.

Bone sialoprotein (BSP) is a mineralized tissue-specific noncollagenous protein that is glycosylated, phosphorylated and sulfated. The temporo-spatial deposition of BSP into the extracellular matrix of bone, and the ability of BSP to bind type I collagen and to nucleate hydroxyapatite crystal formation, indicates a potential role for BSP in the initial mineralization of bone, dentin and cementum. BSP knockout (-/-) mice showed impairment of bone growth and mineralization, concomitantly with dramatical reduction of bone formation. Moreover, BSP has been associated with mineral crystal formation in several pathologies, including breast and prostate carcinomas. The interesting finding of the present study was the

dramtically high expression of BSP in the group with intermittent high extracellular glucose at day 16 and day 22 which was corresponded to an increase in mineralized nodules. Recent study of the overexpression of BSP in MC3T3-E1 cells demonstrated an increase in several osteoblastic markers as well as mineralized nodule formation. The BSP might serve as a matrix-associated signal directly promoting osteoblast differentiation resulting in the increased matrix mineralization.

Vascular calcification is common in diabetes. Bovine vascular smooth muscles incubated with high glucose (25 mM) demonstrated an increase in bone matrix protein expression including BSP and significantly enhanced calcification in a time-dependent manner. Our study showed a significant increase in mineralized nodules at day 29 under the intermittent high extracellular glucose (25 mM alternatively with 5.5 mM every 2 days). This greater nodule formation is corresponding to an increase in cbfa1 expression at day 16 and an increase in BSP expression at day 16 and day 22, a marker of osteoblastic differentiation.

There have been no previous studies investigating the effect of nacre WSM on osteoblasts in the high extracellular glucose condition. In the present study, we have studied the effect of nacre WSM on bone matrix expression and mineralization of MC3T3-E1 cells. Our results demonstrate that nacre WSM has a trend to enhance the matrix mineralization of osteoblastic cell line (MC3T3E1) in the normal medium condition (without extracellular glucose). However, in the presence of extracellular glucose, no significant effect of WSM was found on matrix mineralization.

Conclusion:

The water-soluble matrix of nacre could enhance matrix mineralization produced by the osteoblastic cell line under normal glucose level. Under the intermittent high extracellular glucose, matrix mineralization was delayed but it was accelerated in late mineralization with highly expressed bone sialoprotein. This study demonstrates that the nacre WSM contains signaling molecules which may be able to partially increase osteoblastic activity in high glucose condition.

Abstract

Project Code : MRG4980025

Project Title : Effects of nacre water-soluble matrix on osteoblasts in the elevated extracellular glucose condition

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Project Period : July 1st, 2006 – December 31st, 2008

Nacre is a calcified structure that forms the lustrous inner layer of some molluskan shells. Its organic matrix has been classified as “water-soluble matrix, WSM” and “water-insoluble matrix”, based on its solubility in aqueous solutions. The WSM of nacre is believed to be intimately involved in guiding crystal nucleation and growth through molecular interactions between matrix macromolecules and minerals. In diabetic bone, the osteoblastic bone deposition is decreased whereas the osteoclastic bone resorption is increased resulting in delay matrix mineralization. The purpose of this study was to evaluate whether the WSM from the nacre of *Pinctada maxima* enhances the *in vitro* osteoblastic mineralization in the diabetic condition. MC3T3-E1, an osteoblastic cell line, and primary human bone cells were cultured under normal and intermittent high extracellular glucose conditions for 4 weeks. The mineralized nodules were determined by Alizarin Red S staining and the bone matrix markers were analyzed by real-time PCR. With the presence of the WSM in normal medium condition, MC3T3-E1 cells produced slightly more matrix mineralization than that of control. At day 29, under the intermittent high extracellular glucose condition, the mineralized nodules were more abundant and the bone sialoprotein expression significantly increased, compared to those groups without glucose. However, the effect of WSM under diabetic condition was not significantly different from those of the controls. In conclusion, nacre WSM could enhance matrix mineralization of osteoblastic cell line under normal glucose level. Under the intermittent high extracellular glucose, matrix mineralization was delayed but it was accelerated in the late mineralization with highly expressed bone sialoprotein, a marker of osteoblastic differentiation.

Keywords : nacre of *Pinctada maxima*, mineralization, osteoblast, diabetes, high glucose

บทคัดย่อ

รหัสโครงการ: MRG4980025

ชื่อโครงการ: ผลของเปลือกหอยส่วนที่ละลายน้ำได้ต่อเซลล์สร้างกระดูกในสภาวะที่มีน้ำตาลสูง

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ระยะเวลาโครงการ: ๑ กรกฎาคม ๒๕๔๙ ถึง ๓๑ ธันวาคม ๒๕๕๑

อินทรียสารของชั้นในของเปลือกหอยมุก (nacre) สามารถแบ่งตามคุณสมบัติในการละลายได้เป็น ๒ ส่วน คือ ส่วนที่ละลายน้ำได้ และส่วนที่ไม่ละลายน้ำ ส่วนที่ละลายน้ำได้นั้นเชื่อว่าเป็นตัวนำในการเกิดนิวเคลียส และ การเติบโตของผลึกอนินทรีย์ของเปลือกหอยโดยผ่านอันตรกิริยาเชิงโมเลกุลระหว่างเมทริกซ์แมโครโมเลกุลกับแร่ ในสภาวะเบาหวานการสร้างกระดูกลดลงและการสลายกระดูกเพิ่มขึ้นยังผลให้การสะสมแร่ในกระดูกช้าลง การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความสามารถของส่วนที่ละลายน้ำได้ของชั้นในของเปลือกหอยมุก *(Pinctada maxima)* เพื่อเร่งการสร้างกระดูกในสภาวะที่เป็นเบาหวานได้หรือไม่ โดยทดสอบกับสายพันธุ์ของเซลล์สร้างกระดูก (MC3T3-E1 cell line) และเซลล์สร้างกระดูกปฐมภูมิ (primary bone cells) ที่เลี้ยงในอาหารเลี้ยงเซลล์ที่มีระดับน้ำตาลสูงเป็นระยะๆเทียบกับที่มีระดับน้ำตาลระดับปกติเป็นเวลา ๔ สัปดาห์ แล้วศึกษาการสะสมของแคลเซียมด้วยวิธีย้อมสีAlizarin Red S และวิเคราะห์ตัวบ่งชี้การสร้างกระดูก (bone matrix markers) ด้วยวิธี real-time PCR จากผลการทดลองพบว่าเซลล์สร้างกระดูกที่เลี้ยงในอาหารเลี้ยงเซลล์ที่มีส่วนที่ละลายน้ำได้ของชั้นในของเปลือกหอยมุกอยู่สามารถสะสมแคลเซียมได้มากกว่าปกติ ส่วนในสภาวะที่อาหารเลี้ยงเซลล์มีระดับน้ำตาลสูงเป็นระยะๆ ในวันที่ ๒๙ ของการเพาะเลี้ยงพบมีการสะสมของแคลเซียมมากกว่าปกติ และมีการแสดงออกของ bone sialoprotein สูงกว่าปกติอย่างเห็นได้ชัด แต่ผลของส่วนที่ละลายน้ำได้ของชั้นในของเปลือกหอยมุกต่อการสะสมแคลเซียมยังไม่ชัดเจนใน
สภาวะที่อาหารเลี้ยงเซลล์ที่มีระดับน้ำตาลสูง การวิจัยนี้สรุปได้ว่าส่วนที่ละลายน้ำได้ของชั้นในของเปลือกหอยมุกสามารถเร่งการสะสมของแคลเซียมของเซลล์สร้างกระดูกได้มากกว่าปกติในสภาวะที่มีระดับน้ำตาลปกติ และในสภาวะที่มีน้ำตาลสูงเป็นระยะๆ การสะสมแคลเซียมของเซลล์สร้างกระดูกในระยะแรกช้าลง แต่กลับมีจำนวนมากขึ้นในระยะหลังซึ่งสัมพันธ์กับการมีแสดงออกของ bone sialoprotein ที่สูงกว่าปกติซึ่งเป็นตัวบ่งชี้ในการพัฒนาของเซลล์สร้างกระดูก (osteoblastic differentiation)

คำหลัก: ชั้นในของเปลือกหอยมุก, การสะสมแคลเซียม, เซลล์สร้างกระดูก, เบาหวาน, น้ำตาลสูง

Part I

Title : Effects of nacre water-soluble matrix on MC3T3-E1 in the elevated extracellular glucose condition

Introduction

Diabetes is diagnosis by an elevated blood glucose level that is more than 200 mg/dl at random testing.[1] Cellular response to elevated extracellular glucose may contribute to the development of diabetic complications including increased risk of osteoporosis in the insulin-dependent diabetes mellitus (type I) patients.[2-4] In diabetic bone, the number of fully mature osteoblasts is decreased suggesting that hyperglycemia can suppress osteoblast differentiation.[2, 5] Moreover, glucose has been shown to have a direct activating effect on osteoclasts and seems to be the principal energy source for osteoclastic bone resorption.[6] Recent studies of the MC3T3-E1 osteoblastic diabetic models have shown that an elevation in extracellular glucose levels significantly inhibits calcium uptake[7] and downregulates osteocalcin expression (a marker of differentiated osteoblasts).[8]

Nacre (mother-of-pearl) is a calcified structure that forms the lustrous inner layer of some molluskan shells. Like bone, nacre is produced by depositing mineral onto an organic matrix. Its microarchitecture is described as a “brick-bridge-mortar” arrangement, where the bricks refer to flat polygonal calcium carbonate crystals of aragonite and the mortar is a biological organic adhesive composed of proteins, glycoproteins, polysaccharides and lipids.[9] Nacre organic matrix has been classified as “water-soluble matrix” (WSM) and “water-insoluble matrix”, based on its solubility in aqueous solutions. The WSM of nacre is believed to be intimately involved in guiding crystal nucleation and growth through molecular interactions between matrix macromolecules and mineral.[10]

Several *in vitro* and *in vivo* studies shown that nacre is biocompatible, biodegradable, and osteoconductive.[11-14] When nacre is implanted in bone, new bone formation occurs without any intervening fibrous tissue.[15-17] Furthermore, nacre could induce human dental pulp cells[18] and human periodontal ligament cells[19] to form mineralized nodules. These activities of nacre could be due to its WSM. Recent studies of the nacre WSM have shown that the WSM increases alkaline phosphatase activity of rat bone marrow stromal cells,[13] induces cell differentiation of human fetal lung fibroblasts,[20] stimulates cytoplasmic Bcl-2 production, an anti-apoptotic protein, in rat primary cultured osteoblasts,[14] and produces earlier mineralization of MC3T3-E1 mouse osteoblastic cell line.[21] However, very little is

known about the effect of nacre WSM on osteoblast in diabetic condition. Thus, the objective of the present study was to determine the effect of nacre WSM from *Pinctada maxima* on the mineralization of MC3T3-E1 osteoblastic cell line in the elevated extracellular glucose conditions.

Materials and methods

Extraction and purification of nacre water-soluble matrix

Nacre was obtained from the inner shell layer of the giant oyster *Pinctada maxima* through a non-decalcifying process. Nacre was pulverized until the particle size less than 90 µm using a planetary mill (Retsch S1000, Hann, Germany) and a Vibratory sieve shaker (Fritsch Analysette 3 Pro, Mainbernheim, Germany). Powdered nacre was suspended in distilled water for 24 hour at 4°C, with continuous stirring. The suspension was centrifuged for 20 minutes at 3,500 rpm. The supernatant, called the water-soluble matrix (WSM), is then lyophilized.

Cell culture conditions

The MC3T3-E1 mineralizing subclone (subclone 4) from American Type Culture Collection was plated onto 35 mm dishes with plate density 10^4 cells/cm^2 and was grown in alpha minimal essential medium (α-MEM) containing 10% fetal bovine serum supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B in a 5% CO_2 atmosphere at 37°C. Media were changed every 2 days.

Determination of the concentration of nacre WSM extract

To determine the suitable concentration of nacre WSM extract and culture conditions, MC3T3-E1 cells (MC) were cultured until confluence and were divided into 6 groups as follow: (1) MC; (2) MC and 135 µg/ml WSM; (3) MC and 200 µg/ml WSM; (4) MC, 50 µg/ml ascorbic acid (AA) and 2 mM β-glycerophosphate (βGP); (5) MC, 50 µg/ml AA, 2 mM βGP, and 135 µg/ml WSM; (6) MC, 50 µg/ml AA, 2 mM βGP, and 200 µg/ml WSM. The media were changed every 2 days for 4 weeks. The mineralized nodule formation was observed under light microscope.

Cell culture in the presence of WSM and extracellular glucose

MC3T3-E1 cells (MC) were cultured until confluence and were divided into 6 groups as follow: (1) MC; (2) MC, 50 µg/ml AA, 2 mM βGP; (3) MC, 50 µg/ml AA, 2 mM βGP, and

135 μg/ml WSM; (4) MC and glucose; (5) MC, 50 μg/ml AA, 2 mM βGP, and glucose; (6) MC, 50 μg/ml AA, 2 mM βGP, 135 μg/ml WSM and glucose. Group 1-3 were incubated in normal α-MEM medium (containing 5.5 mM D-glucose) and Group 4-6 were incubated in normal and high glucose (25 mM D-glucose) media alternating every 2 days. MC cells only (Group 1) was used as a negative control.

In vitro mineralization assay

Cell culture of diabetic models and the controls were stained with Alizarin red S at 1, 2, 3, 4, 5 weeks according to previous protocol.[22] Briefly, cell layers/matrices were washed with phosphate buffer saline twice, fixed with 100% methanol, and stained with 1% Alizarin red S for 1 minute. The cell layers/matrice were then washed with distilled water and air dried. At 3 and 4 weeks, the mineralized areas per visual fields (randomly selected five per dish) were analyzed by using Scion image software and calculated as the percentages of mineralized areas to total areas.

Bone matrix gene expression analysis

Cell culture of diabetic models and the controls were collect at 1, 2, 3 weeks for bone matrix gene expression analysis. Real-time polymerase chain reaction (PCR) of osteoblastic markers including core binding factor alpha subunit 1 (Cbfa1) and collagen type I alpha 2 chain (Col1a2) were analyzed at 1 and 2 weeks. The osteocalcin (OC) and bone sialoprotein (BSP) were analyzed at 2 and 3 weeks. GAPDH was used as an internal control. Briefly, total RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed on 1 μg total RNA in the initial mix with Quantitect reverse transcriptase according to the manufacturer's instructions (Quantitect®, Qiagen Inc., Valencia, CA, USA). Relative quantitative real-time PCR was performed using sequence specific primers and the ABI 7500 Real time PCR system (Applied Biosystems, Foster city, CA, USA). Primers and probes were obtained commercially as follows: Cbfa1 (ABI assay No. Mm00501578_m1), Col1a2 (ABI assay No. Mm00483888_m1), OC (ABI assay No. Mm01741771_g1), BSP (ABI assay No. Mm00492555_m1), GAPDH (ABI assay No.4308313) and TaqMan® Universal PCR master mix (Applied Biosystems, Foster city, CA, USA). The amplification was done under the following conditions: 50°C, 2 min; 95°C, 10 min; followed by 40 cycles of 95°C, 15 s and 60°C, 1 min. Data were analyzed using the ABI Prism 7500 1.3.1 software (Applied Biosystems, Foster city, CA, USA).

Results

Suitable concentration of nacre WSM extract for MC3T3-E1

MC3T3-E1 cell cultures containing 50 µg/ml AA, 2 mM βGP, and either 135 µg/ml WSM or 200 µg/ml WSM showed cell accumulation as an initial site of nodule formation at day 18 under the light microscope. The former produced nodule larger and more abundant than the latter. MC3T3-E1 cell cultures containing 50 µg/ml AA, 2 mM βGP showed the sites of nodule formation at day 28 (Fig.1). The other three groups without AA and βGP: MC; MC and 135 µg/ml WSM; MC and 200 µg/ml WSM, were not produced nodules at day 28 (Data not shown).

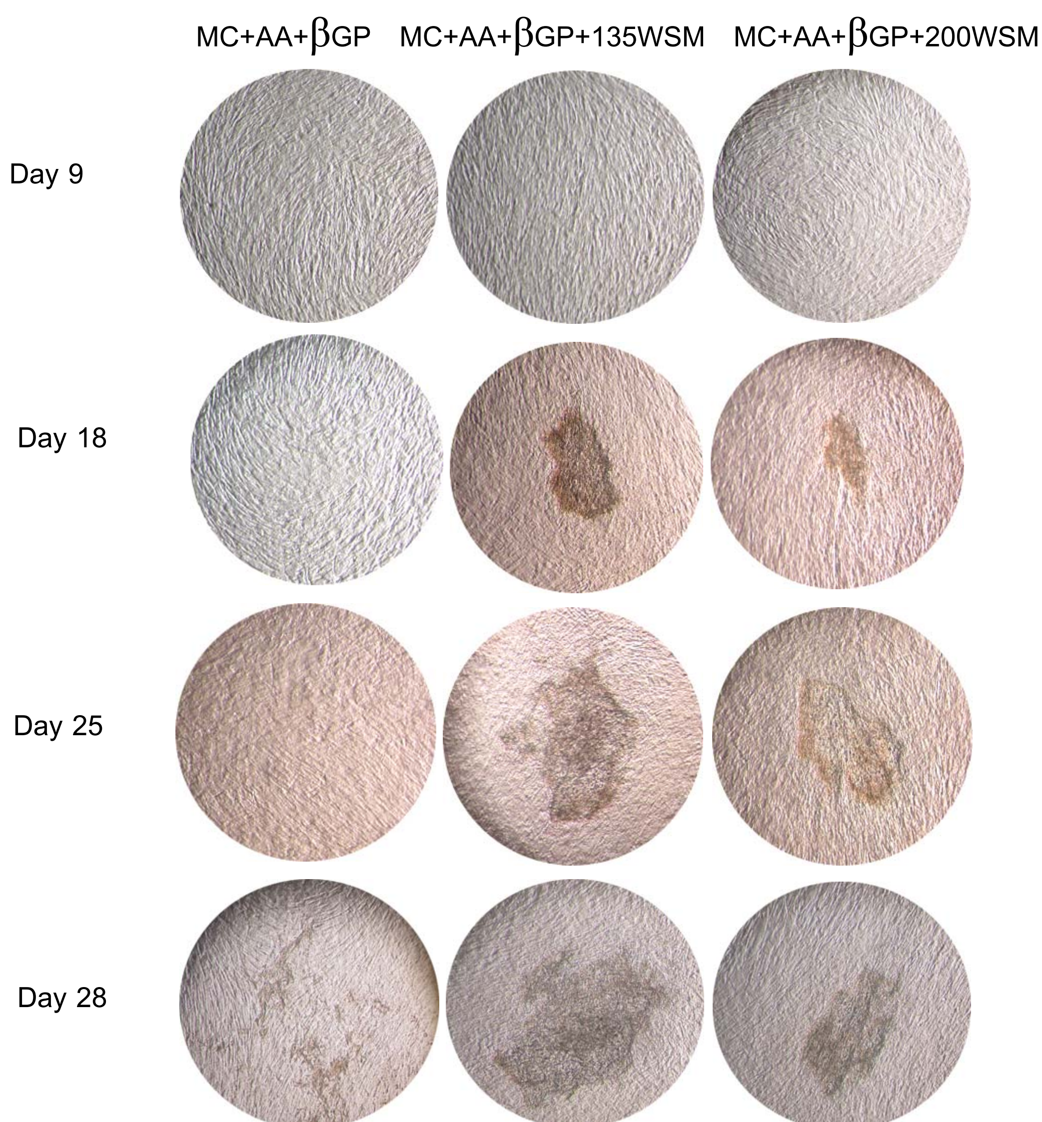

Fig.1 Phase-contrast micrographs (x100) to determine the suitable concentration of nacre WSM and conditions for matrix mineralization.

Glucose delayed the matrix mineralization

At 2 and 3 weeks, MC3T3-E1 cell cultures containing 50 µg/ml AA, 2 mM βGP and intermittent high glucose produced mineralized nodules less than those groups without intermittent high glucose. Although the groups with intermittent high glucose increased in nodule formation at 4 and 5 weeks, this formation is still less than the groups without glucose (Fig.2).

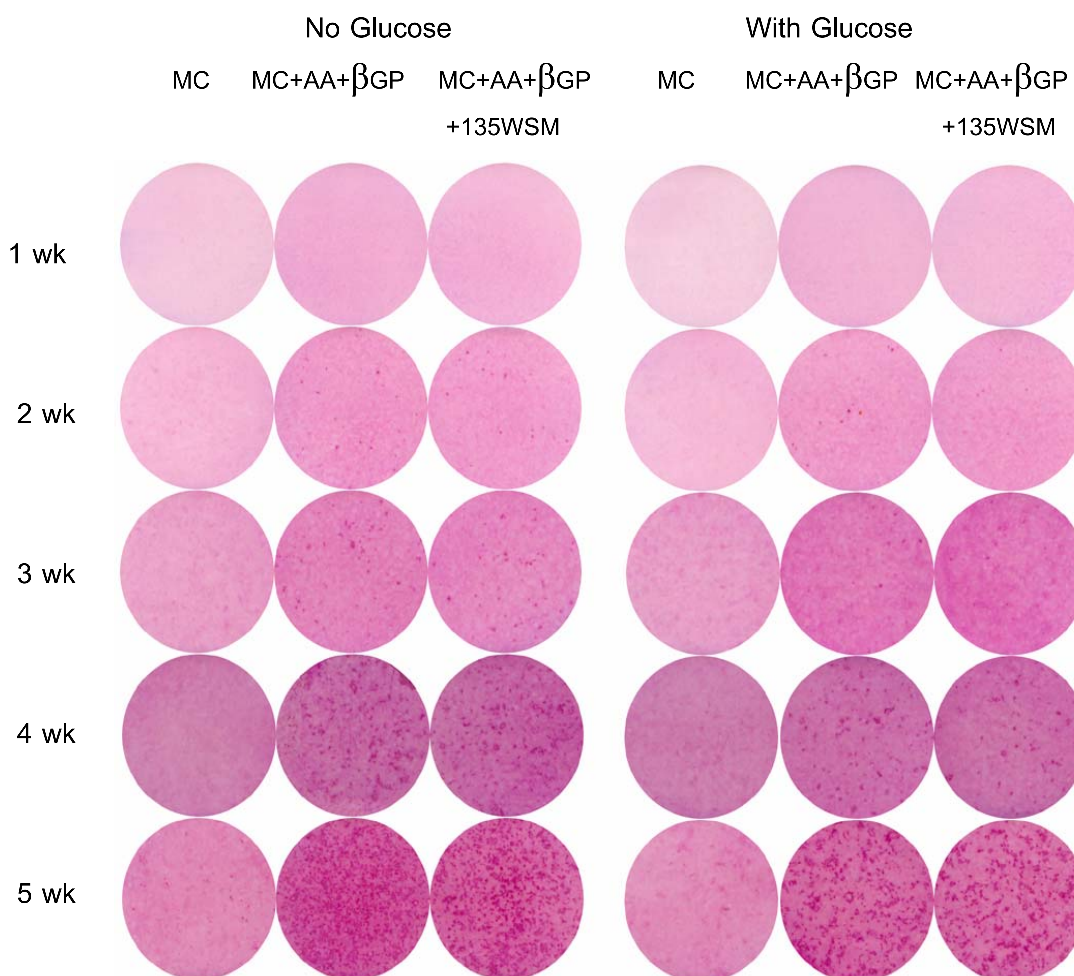

Fig.2 Alizarin Red S staining of matrix mineralization from 1-5 weeks

WSM did not significantly enhance nodule formation in the elevated extracellular glucose condition

At 2 and 3 weeks, MC3T3-E1 cell cultures containing 50 µg/ml AA, 2 mM βGP, 135 µg/ml WSM and intermittent high glucose did not produce mineralized nodules more than the group without WSM (Fig. 2, 3). At 4 week, MC3T3-E1 cell cultures containing 50 µg/ml AA,

2 mM βGP and 135 µg/ml WSM produced nodules similar to the group without WSM. MC3T3-E1 cell cultures containing 50 µg/ml AA, 2 mM βGP, 135 µg/ml WSM and intermittent high glucose produced nodules similar to the group without WSM (Fig.2, 3).

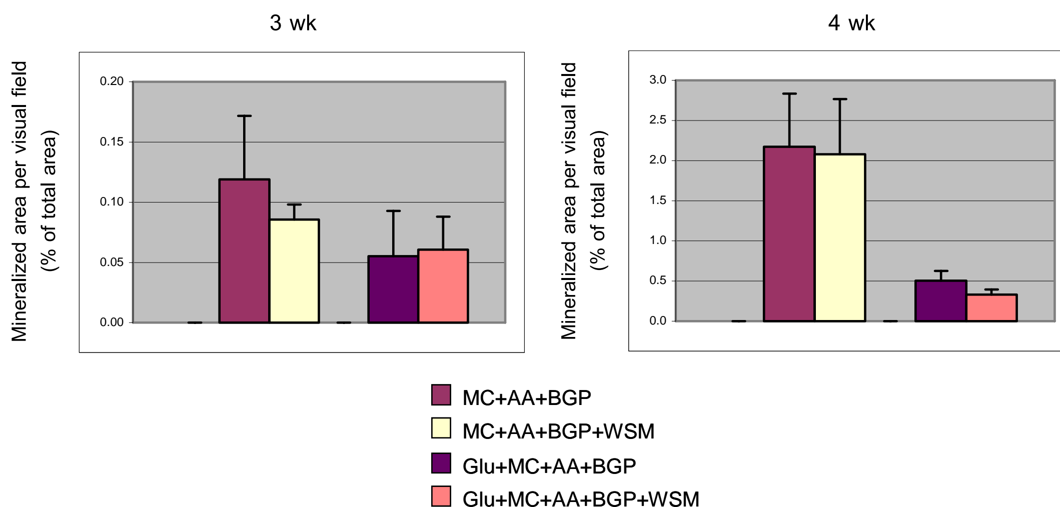

Fig.3 Mineralized nodule areas per visual field calculated as the percentage of mineralized areas to the total areas. Data are shown as the mean ± S.D. of 5 visual fields.

Bone matrix gene expression by relative quantitative real-time PCR

At 1 week of intermittent high glucose condition, MC3T3-E1 cell cultures containing 50 µg/ml AA, 2 mM βGP, with or without 135 µg/ml WSM exhibited higher Cbfa1 and Col1a2 expression than the controls, however; this expression decreased at 2 weeks (Fig. 4A-4D). With intermittent high glucose condition, MC +AA+ βGP+WSM showed less Cbfa1 and Col1a2 expression than MC +AA+ βGP (Fig. 4A-4D).

The OC expression of cultures with intermittent high glucose condition were less than those groups without glucose, however; no different of these OC expression with or without WSM (Fig. 4E, 4G).

At 2 week, the BSP expression of cultures with intermittent high glucose condition was less than those cultures without glucose (Fig. 4F). This BSP expression was dramatically reduced at 3 week (Fig. 4H).

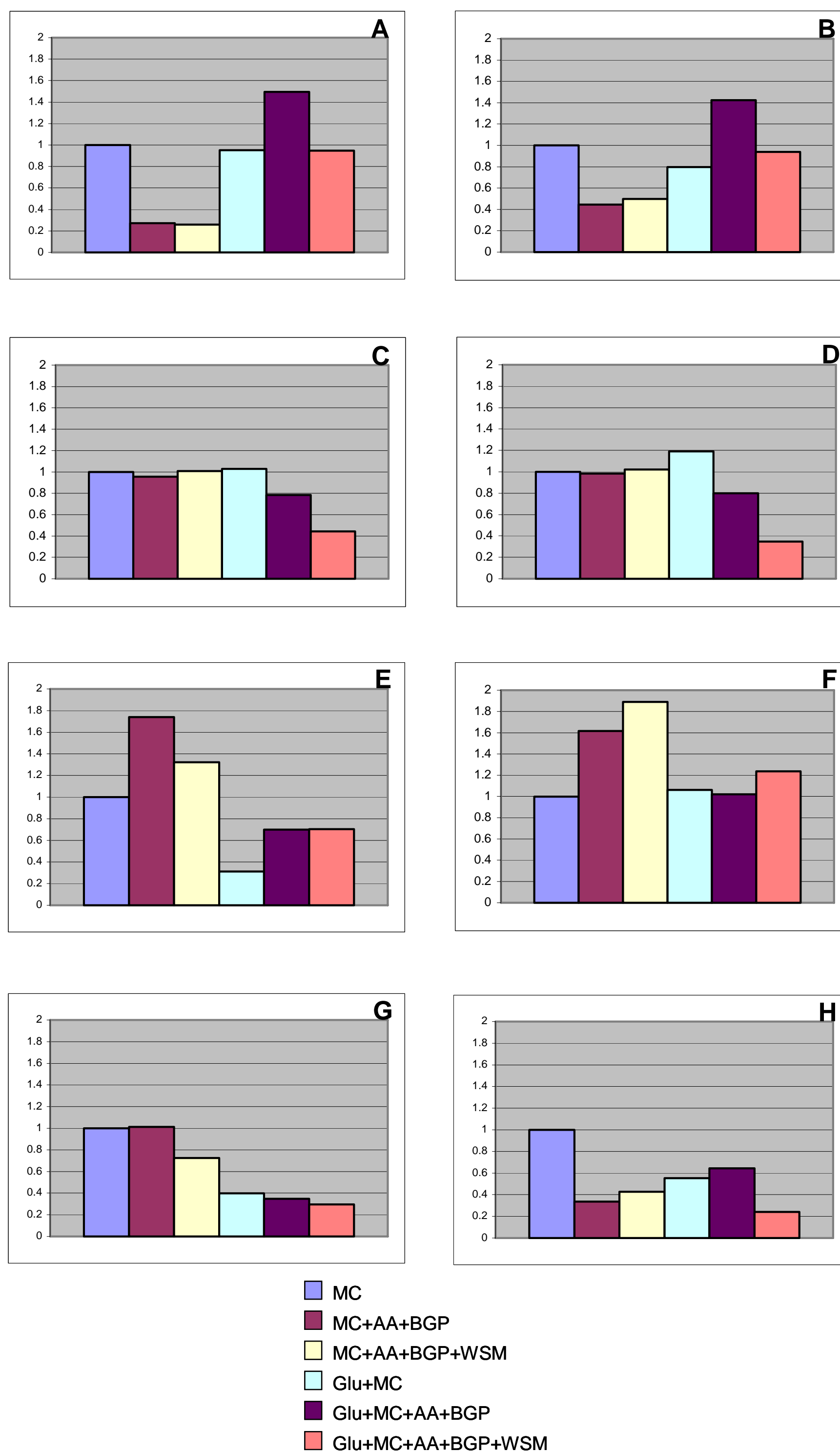

Fig.4 Bone matrix gene expression by relative quantitative real-time PCR: (A) core binding factor alpha subunit 1 at 1 wk; (B) collagen type I alpha 2 chain at 1 wk; (C) core binding factor alpha subunit 1 at 2 wk; (D) collagen type I alpha 2 chain at 2 wk; (E) osteocalcin at 2 wk; (F) bone sialoprotein at 2 wk; (G) osteocalcin at 3 wk; (H) bone sialoprotein at 3 wk.

Discussion

MC3T3-E1 cells is a well-characterized preosteoblastic cell line derived from mouse calvaria.[23] In the presence of 50 μg/ml AA and 2 mM inorganic phosphate, mineralizing subclone (subclone 4) of this cell line formed nodules at day 8 and expressed several osteoblastic markers including OC and BSP.[23] In the present study, MC3T3-E1 cells produced matrix mineralization at day 14, later than the original clone due to repeated passage and nonmineralizing colonies appear.

Glucose concentrations used in the present study correspond to healthy individuals (5.5 mM D-glucose) and to poorly controlled-diabetic patient (25 mM D-glucose, 5x normal). We used the intermittent high glucose (5.5 mM D-glucose alternating with 25 mM D-glucose every 2 days) because this condition exhibited significantly different in MC3T3-E1 cells,[24] cultured rat mesangial cells[25] and cultured human tubulointerstitial cells [26]. Furthermore, the constant high glucose concentrations do not represent the conditions experienced by the cells of diabetic patients. The high glucose concentrations (15 mM or 25 mM D-glucose) have no effect on bone matrix protein as shown by previous comparisons with D-mannitol, providing equivalent osmolarity as D-glucose.[7, 27]

Clinical and *in vivo* studies suggested that diabetes has an effect on bone metabolism leading to osteopenia.[3, 28, 29] Diabetic mice showed diminished expression of osteocalcin, Cbfa1, and collagen type I.[30] *In vitro* studies in the presence of elevated extracellular glucose levels simulating diabetes resulted in a decrease calcium uptake and down regulation of osteocalcin at day 30 of MC3T3-E1 cell culture,[7] a decrease in bone marrow stromal cells proliferation, alkaline phosphatase activity and the number of mineralized nodules formed,[31] a decrease in periodontal cell proliferation and differentiation.[32] Our results clearly demonstrate that under the intermittent high glucose condition, MC3T3-E1 cells had delayed mineralization and reduced the expression of Cbfa1, Col1a2, OC, BSP at 2 weeks, similar to previous studies.[7, 30-32]

Only early stage (1 week), high glucose increased the expression of Cbfa1 and Col1a2 of MC3T3-E1 cell culture. This result is similar to previous studies of human tubulointerstitial cell culture which demonstrated the enhancement of cell growth and collagen synthesis at day 4,[26] vascular smooth muscle cell culture which showed increase the expression of Cbfa1 and BMP-2 at 48 or 72 hours.[27]

There have been no previous studies investigating the effect of nacre WSM on osteoblasts in the high extracellular glucose condition. In the present study, we have studied the effect of nacre WSM on bone matrix expression and mineralization of MC3T3-E1 cells.

Our results demonstrate that nacre WSM did not significantly enhance the matrix mineralization in the high extracellular glucose condition. It is possible that the process of nacre extraction may not suitable including heating during pulverized nacre. Another explanation is the action of nacre WSM may be decreased in the presence of high extracellular glucose. This could be due to the interaction between glucose and nacre WSM. Furthermore, the source of *Pinctada maxima* and the extraction of nacre WSM were different from the previous studies.[20, 21] Rousseau *et al* reported that the nacre of *Pinctada maxima* produced earlier mineralization of MC3T3-E1 cells, their study used 10 mM βGP, stained nodules at day 6, and showed only phase-contrast micrographs.[21] Mouriés *et al* showed that the nacre WSM from *Pinctada maxima* increased significantly alkaline phosphatase activity of rat bone marrow cell cultures at day 1 but the response was not dose-dependent (135, 270, 540 μg/ml WSM).[20] The nacre WSM seems to stimulate the proliferation stage of the osteoprogenitor cells.

Acknowledgments

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Part II

Title : Effects of nacre water-soluble matrix and EDTA-extract on primary human bone cells in the elevated extracellular glucose condition.

Introduction

Nacre organic matrix has been classified as both 'water-soluble' and 'water-insoluble', based on its solubility in aqueous solutions after decalcification with acid or EDTA.[1] Several studies have reported the usefulness of water-soluble matrix including formation of new bone without any intervening fibrous tissue,[2-4] induction human dental pulp cells[5] and periodontal ligament cells[6] to form mineralized nodules. The water-insoluble matrix or EDTA extract contains glycosaminoglycans (GAGs) which are less in water-soluble matrix.[1] The role of GAGs is not really understood, but their presence in the organic matrices of bones, teeth and avian eggshell shows their importance in biomineralizing systems.[1] Furthermore, proteoglycans which are GAGs associated to a core protein may act in cell signaling and metabolic activity.[7]

Another model to study the osteoblastic differentiation is primary bone cell culture. Primary cell culture is derived either by the outgrowth of migrating cells from a fragment of tissue or by enzymatic or mechanical dispersal of the tissue. It is the first in a series of selective processes that may ultimately give rise to a relatively uniform cell line.

However, very little is known about the effect of nacre water-soluble matrix and EDTA extracts on osteoblast in diabetic condition. Thus, the objective of the present study was to determine the effect of nacre water-soluble matrix and EDTA-extract from *Pinctada maxima* on the mineralization of human primary bone cells in the elevated extracellular glucose condition.

Materials and methods

Extraction and purification of nacre water-soluble matrix

Two shells of *Pinctada maxima* were obtained. The white-inner shell layer of the pearl, called nacre, was separated from its shell using a hammer. Several pieces of nacre were then crushed using a pestle and mortar. The nacre chips were then suspended in distilled water for 20 hour, centrifuged for 20 minute at 3,500 rpm, and the supernatant was lyophilized and collected as the water-soluble matrix (WSM). The known amounts of

lyophilized powder was suspended in sterile-distilled water and filtered with 0.2 micron syringe filter. The final concentration of WSM is 135 μg/ml.

Extraction and purification of EDTA-extract from nacre

The remaining nacre chips after water extraction were demineralized with 0.5M EDTA, pH 7.4 with continuous stirring for 7 days. The supernatant was intensively dialyzed against distilled water and lyophilized. The precipitate was demineralized with 0.5 M EDTA again for 7 days. The supernatant was dialyzed against distilled water, combined with the first supernatant, and lyophilized. The known amounts of EDTA extract-lyophilized powder was suspended in sterile-distilled water and filtered with 0.2 micron syringe filter. The final concentration of EDTA extract is 135 μg/ml.

Human primary bone cells culture

Human bone was obtained from a patient who was received jaw resection for orthodontic treatment. The periosteum and soft tissues were carefully removed. The bone was washed with 0.01 M phosphate-buffer saline (PBS), pH7.2 for 5-7 times and then digested with collagenase 1 mg/ml in Hank's B solution twice, at 37°C for 1 hour. The bone was chopped into small pieces, washed with 0.01 M PBS, centrifuged at 1500 rpm for 10 minutes, and discarded supernatant. The pieces of bone were placed onto two 25 cm^2 culture flasks, containing alpha minimal essential medium (α-MEM), 10% fetal bovine serum supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. The culture flasks were incubated in a 5% CO_2 atmosphere incubator, at 37°C. Media were changed every 2-3 days. Cells migrated out from the bone fragments were released from the surface with 0.25% trypsin /EDTA (5 min, at 37°C) and media were added to stop trypsinization. After centrifugation, the cells were plated onto six 75 cm^2 culture flasks, and defined as first passage cells. After growth to 100% confluence, the cells were released from the culture flasks as described above, counted and plated onto one hundred and forty-eight dishes of 35 mm culture dishes at a density of 10^4 cells/cm^2. The media were α-MEM containing 10% fetal bovine serum supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B in a 5% CO_2 atmosphere at 37°C and were changed every 2-3 days. The 142 culture dishes were divided into three experiments: 12 dishes for alkaline phosphatase activity, 64 dishes for nodule formation, and 72 dishes for real-time PCR.

Cell culture in the presence of WSM or EDTA extract under intermittent high extracellular glucose condition

Primary human bone cells (BC) were cultured until confluence and were divided into 8 groups as follow (each group has seventeen 35 mm culture dishes): (1) BC; (2) BC, 50 µg/ml AA, 2 mM β-glycerophosphate (βGP); (3) BC, 50 µg/ml AA, 2 mM βGP, and 135 µg/ml WSM; (4) BC, 50 µg/ml AA, 2 mM βGP, and 135 µg/ml EDTA extract; (5) BC and glucose; (6) BC, 50 µg/ml AA, 2 mM βGP, and glucose; (7) BC, 50 µg/ml AA, 2 mM βGP, 135 µg/ml WSM and glucose; (8) BC, 50 µg/ml AA, 2 mM βGP, 135 µg/ml EDTA extract and glucose;. Group 1-4 were incubated in normal α-MEM medium (containing 5.5 mM D-glucose) and Group 5-8 were incubated in normal and high glucose (25 mM D-glucose) media alternating every 2 days. BC cells only (Group 1) was used as a negative control.

Alkaline phosphatase activity

The cell layers were collected from three 35 mm culture dished at each time point of day 3, 6, 9, 12 after plate. The cell layers were washed with 0.01 M PBS three times, lysed with 150 µl of lysis buffer. The 20 µl of suspension was used for measurement of alkaline phosphatase activity. The reaction is started by adding 100 µl of 125 mM carbonate bicarbonate buffer, pH 10.3 at 37°C for 5 min. The samples were then mixed with 100 µl of 2 mM $MgCl_2$, 2 mM p-nitrophenylphosphate and incubated at 37°C for 15 min. The reaction is stopped by adding 100 µl of 0.2 M NaOH. The liberated p-nitrophenol was measured spectrophotometrically at 405 nm.

In vitro mineralization assay

Cell culture of diabetic models and the controls were stained with Alizarin red S at 1, 2, 3, 4, 5, 6, 7 weeks according to previous protocol.[8] Briefly, cell layers/matrices were washed with PBS twice, fixed with 100% methanol, and stained with 1% Alizarin red S for 1 minute. The cell layers/matrice were then washed with distilled water and air dried.

Bone matrix gene expression analysis

Cell culture of diabetic models and the controls were collect at 1, 2, 3, 5 weeks for bone matrix gene expression analysis including core binding factor alpha subunit 1 (Cbfa1), collagen type I alpha 2 chain (Col1a2), osteocalcin (OC) and bone sialoprotein (BSP). The samples were collected by adding Trizol to the cell layers and kept at -80°C for RNA

extraction and RT-Real-time polymerase chain reaction. GAPDH was used as an internal control.

Results

Primary human cell culture

Bone cells were emerged from bone explant at day 7. The phase-contrast micrographs of human primary bone cells at day 16 and day 23 after placed onto two 25 cm^2 culture flasks, are shown in Fig. 1 and 2, respectively.

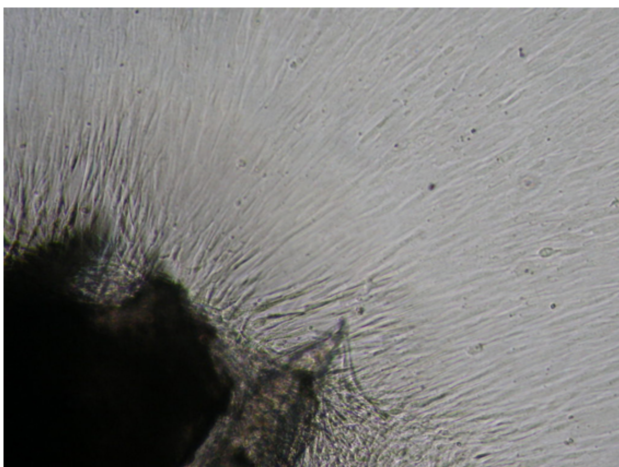

Fig. 1 Bone cell explants at day 16 (x100 magnification)

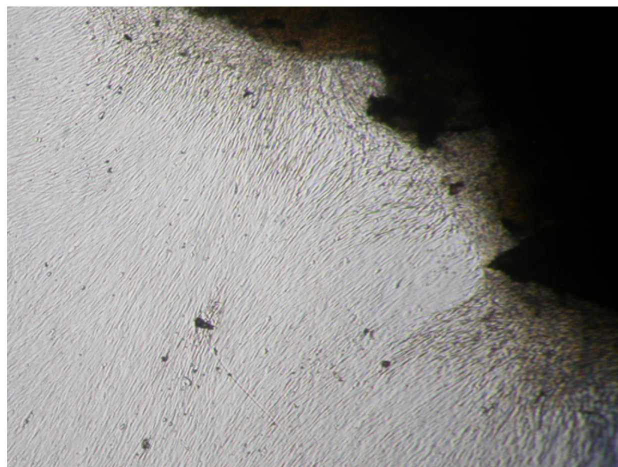

Fig. 2 Bone cell explants at day 23 (x100 magnification)

Alkaline phosphatase activity

Human primary bone cells were plated at the same time as the tests for nodule formation and bone matrix gene expression. Cell layers were collected from three 35 mm culture dishes at day 3, 6, 9, 12 after plate. They were then washed with PBS three times and kept at -20°C for measurement of alkaline phosphatase activity. However, due to the minimal amounts of nodule formation in this experiment, we did not continue measuring the alkaline phosphatase activity.

Nodule formation of primary human bone cells: Phase-contrast microscopy

The nodule formation was initiated at 2 weeks after confluence. The groups with extracellular glucose produced small sizes of nodules than those without sugar (Fig. 3). The culture with the presence of nacre WSM produced slightly larger nodules that those with EDTA extract. With the presence of EDTA extract, the nodule formation did not differ from the positive control (BC +AA + βGP), as shown in Fig. 3.

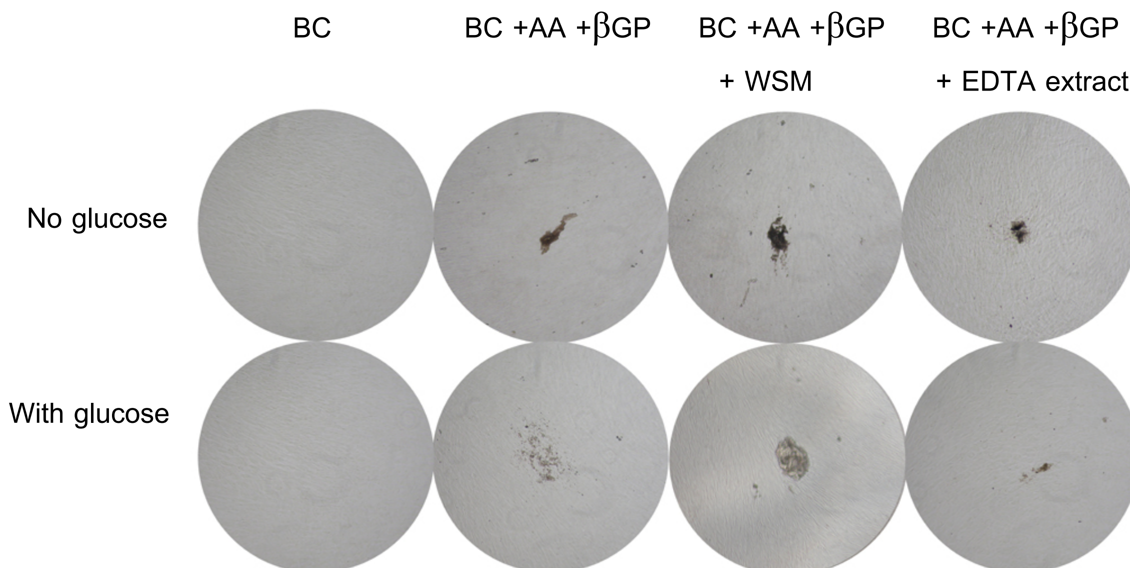

Fig. 3 Phase contrast micrographs (x100) of nodule formation at 2 weeks

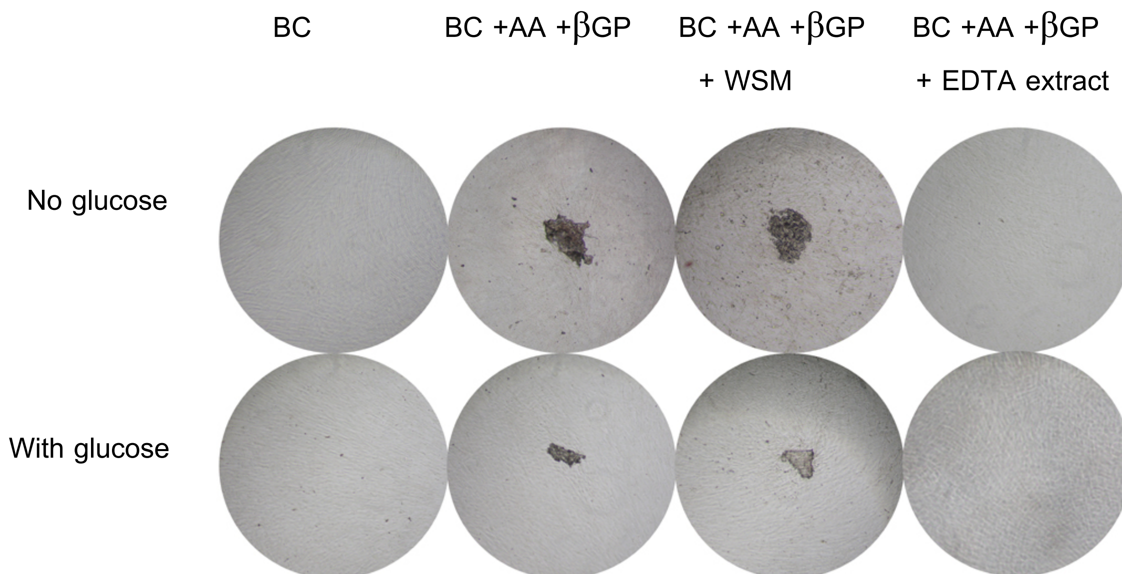

Fig. 4 Phase contrast micrographs (x100) of nodule formation at 4 weeks

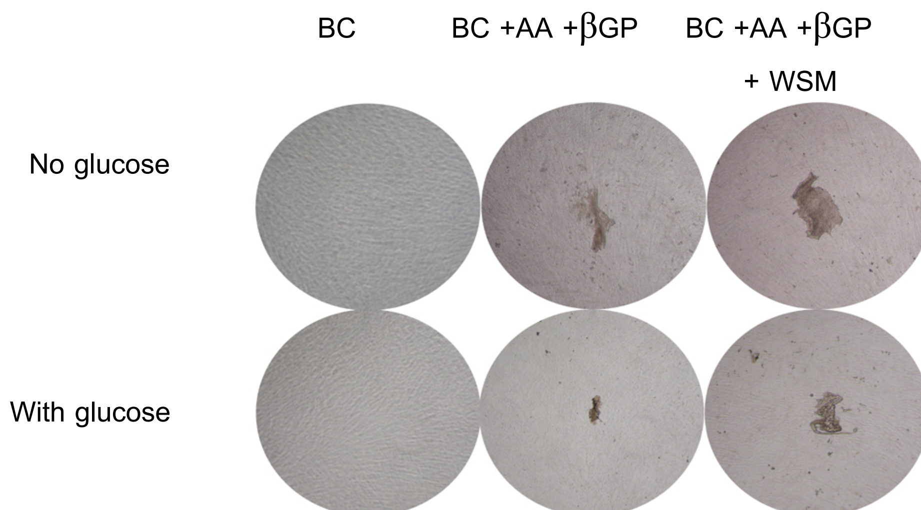

Fig. 5 Phase contrast micrographs (x100) of nodule formation at 5 weeks

At 4 weeks, the group with glucose produced smaller nodules than those without glucose (Fig. 4). With the presence of WSM, the nodule formation did not differ from the positive control without WSM, however, it was better than with EDTA extract which showed no nodules.

At 5 weeks, no data of EDTA extract was available due to limitation the amounts of EDTA extract. The nodule formation with or without WSM were not different from those of 4 weeks (Fig. 5).

Nodule formation of primary human bone cells: Alizarin Red S staining

The Alizarin Red S staining of the whole 35 mm culture dishes at 2, 4, 6 weeks are shown in Fig. 6, 7, 8 respectively. Very few nodules were formed even at 6 weeks of culture, both with and without WSM or EDTA extracts.

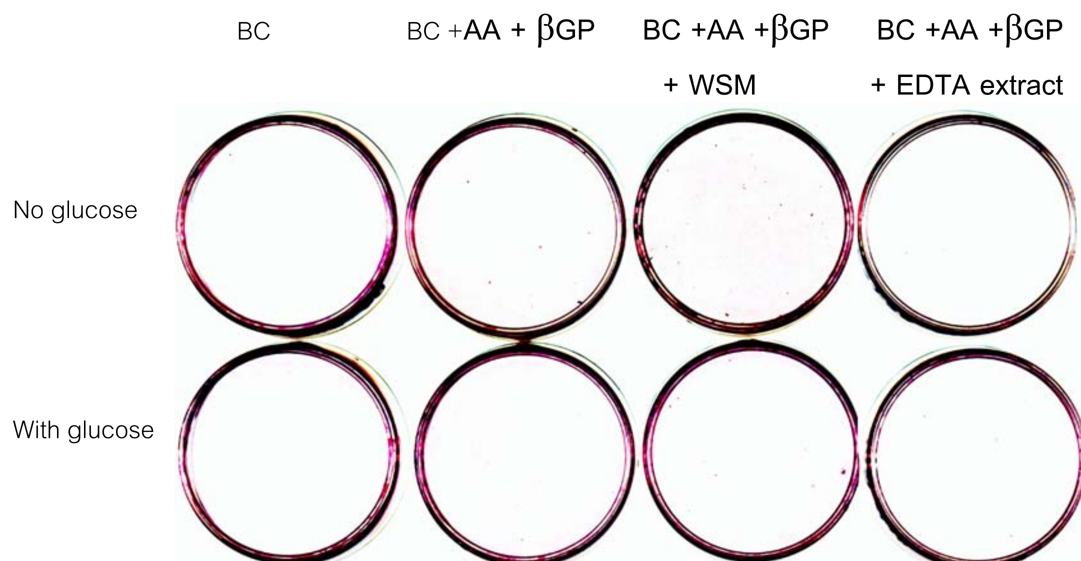

Fig. 6 Alizarin Red S staining of nodule formation at 2 weeks (the whole 35 mm dish)

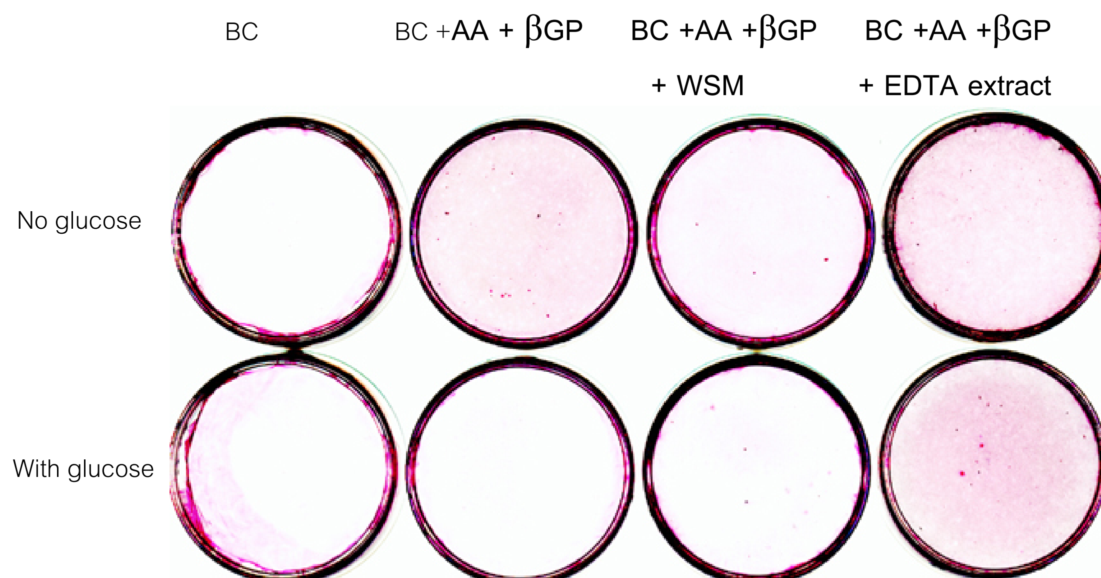

Fig. 7 Alizarin Red S staining of nodule formation at 4 weeks (the whole 35 mm dish)

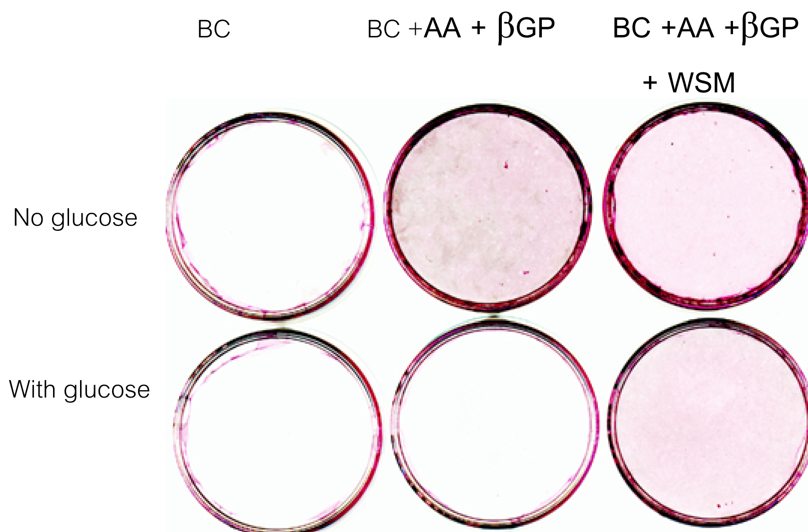

Fig. 8 Alizarin Red S staining of nodule formation at 6 weeks (the whole 35 mm dish)

Discussion

In the present study, we used primary human bone cells to evaluate the effects of nacre WSM or EDTA extract in the extracellular intermittent high glucose media, compared to normal condition. However, the primary bone cells were not be able to produced significant amounts of mineralized nodules even in the normal condition (with ascorbic acid and 2 mM βGP). With the presence of extracellular glucose, the mineralized nodule formation seem to be smaller than without glucose. This smaller nodules produced by primary bone cells was similar to those formed by osteoblastic cell line, MC3T3-E1 (Part I, III). The primary bone cells with water-insoluble matrix (EDTA extract) were not produced more amounts of mineralized nodules than those with water-soluble matrix. Due to limitation in the amounts of EDTA extract, we performed the culture with EDTA extract (group 4, 8) only 4 weeks. Because of no significant mineralized nodules, we did not analyze bone matrix markers.

Acknowledgments

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Part III

Title : Effects of nacre water-soluble matrix on MC3T3-E1 in the elevated extracellular glucose condition

Introduction

Due to the unclear results of Part I, this study was performed to clarify the effects of nacre water-soluble matrix on MC3T3-E1 in the elevated extracellular glucose condition. We changed the methods of nacre extraction by using a pestle and motar because this technique did not produce heat during grinding the nacre chip. In the previous experiment (Part I), we used a planetary mill which produced heat during grinding. The heat might cause denaturing of some important proteins leading to inaccurate result. In the present study, we used triplicate culture dishes for real-time PCR analysis to gain better results of bone matrix gene expression analysis. We used only MC3T3-E1 osteoblastic cell line, not primary bone cells, because MC3T3-E1 cell line produced more amounts of mineralized nodules, compared to those of primary bone cells (Part II). Furthermore, we investigated only water-soluble matrix (WSM) because EDTA extracts seem not to produce better mineralized nodules (Part II).

Materials and methods

Extraction and purification of nacre water-soluble matrix

Two shells of *Pinctada maxima* were obtained. The white-inner shell layer of the pearl, called nacre, was separated from its shell using a hammer. Several pieces of nacre were then crushed using a pestle and mortar. The total amounts of nacre powders were 410 grams (Fig. 1). The nacre powders were then suspended in distilled water (820 ml) for 11 days, centrifuged for 20 minute at 3,500 rpm, and the supernatant was lyophilized and collected as the water-soluble matrix (WSM). The known amounts of lyophilized powder was suspended in sterile-distilled water and filtered with 0.2 micron syringe filter. The working concentration of WSM is 135 μg/ml.

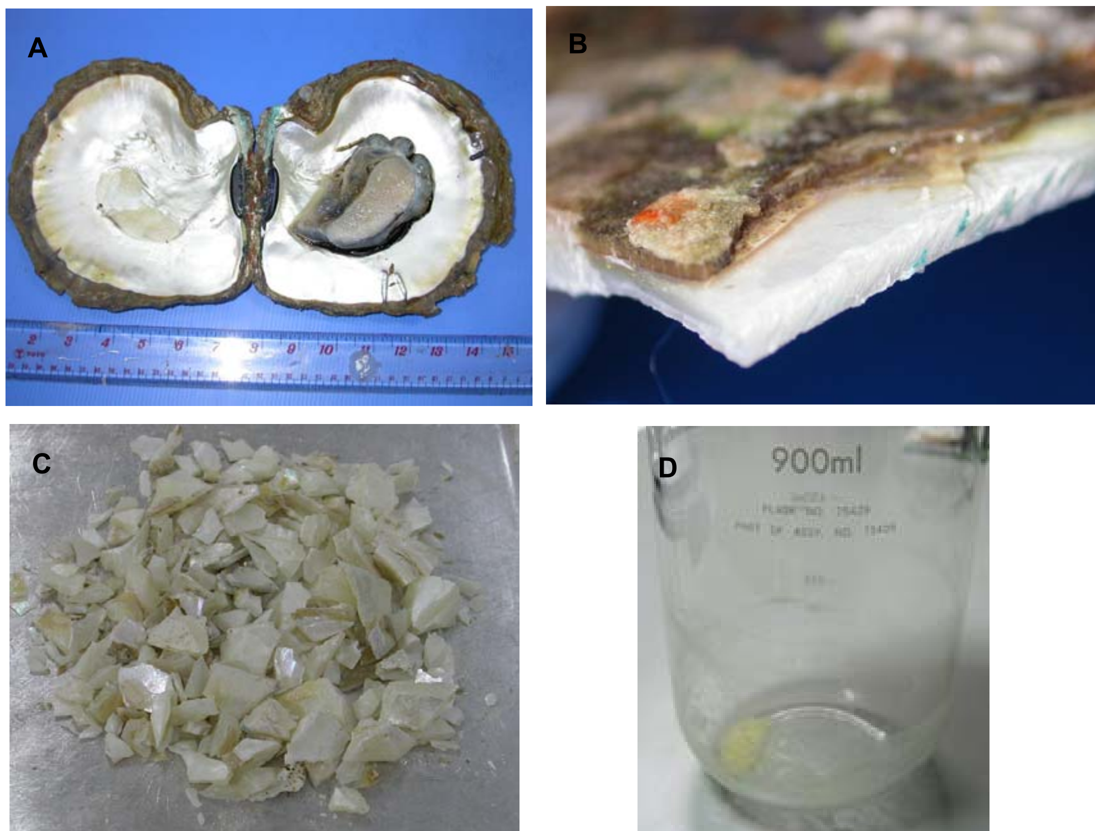

Fig. 1 Preparation of nacre water-soluble matrix (WSM): A, shells of *Pinctada maxima;* B, white-inner shell layer of the pearl, called nacre; C, nacre fragments; D, lyophilized nacre powder.

Cell culture conditions

The MC3T3-E1 mineralizing subclone (subclone 4) from American Type Culture Collection was plated onto 120 dishes (35 mm dish) with plate density 10^4 cells/cm^2 and was grown in alpha minimal essential medium (α-MEM) containing 10% fetal bovine serum supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B in a 5% CO_2 atmosphere at 37°C. Media were changed every 2 days.

Cell culture in the presence of WSM and extracellular glucose

MC3T3-E1 cells (MC) were cultured onto one hundred and twenty 35 mm culture dishes until confluence and were divided into 6 groups as follow: (1) MC; (2) MC, 50 µg/ml AA, 2 mM βGP; (3) MC, 50 µg/ml AA, 2 mM βGP, and 135 µg/ml WSM; (4) MC and glucose; (5) MC, 50 µg/ml AA, 2 mM βGP, and glucose; (6) MC, 50 µg/ml AA, 2 mM βGP, 135 µg/ml WSM and glucose. Group 1-3 were incubated in normal α-MEM medium

(containing 5.5 mM D-glucose) and Group 4-6 were incubated in normal and high glucose (25 mM D-glucose) media alternating every 2 days. MC cells only (Group 1) was used as a negative control.

In vitro mineralization assay

Cell culture of diabetic models and the controls were stained with Alizarin red S at day7, 16, 22, 29 according to previous protocol.[1] Briefly, cell layers/matrices were washed with phosphate buffer saline twice, fixed with 100% methanol, and stained with 1% Alizarin red S for 1 minute. The cell layers/matrice were then washed with distilled water and air dried. The stained 35 mm culture dishes from each group at day 16, 22, 29 were scanned with a flatbed scanner. The mineralized areas were analyzed by using Scion image software. At day 16, there were two dishes per group. At day 22, there were three dishes per group. At day 29, there were five dishes per group. The average mineralized area and the standard deviation of each group was calculated as the percentage to the total culture area of 35 mm dish.

Bone matrix gene expression analysis

Cell culture of diabetic models and the controls were collect from three 35 mm culture dishes per group at day 7, 16, 22, 29 for bone matrix gene expression analysis. Real-time polymerase chain reaction (PCR) of osteoblastic markers including core binding factor alpha subunit 1 (Cbfa1) was analyzed at day 7 and 16. The collagen type I alpha 2 chain (Col1a2) was analyzed at day 7, 16, and 22. The osteocalcin (OC) and bone sialoprotein (BSP) were analyzed day 16 and 22. GAPDH was used as an internal control. Briefly, total RNA was extracted using Trizol® reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed on 1 µg total RNA in the initial mix with Quantitect reverse transcriptase according to the manufacturer's instructions (Quantitect®, Qiagen Inc., Valencia, CA, USA). Relative quantitative real-time PCR was performed using sequence specific primers and the ABI 7500 Real time PCR system (Applied Biosystems, Foster city, CA, USA). Primers and probes were obtained commercially as follows: Cbfa1 (ABI assay No. Mm00501578_m1), Col1a2 (ABI assay No. Mm00483888_m1), OC (ABI assay No. Mm01741771_g1), BSP (ABI assay No. Mm00492555_m1), GAPDH (ABI assay No.4308313) and TaqMan® Universal PCR master mix (Applied Biosystems, Foster city, CA, USA). The amplification was done under the following conditions: 50°C, 2 min; 95°C, 10 min; followed by 40 cycles of 95°C, 15 s and 60°C, 1 min. The relative quantitation (RQ) was

analyzed using the ABI Prism 7500 1.3.1 software (Applied Biosystems, Foster city, CA, USA). The MC3T3-E1 cells only was used as a negative control and calculated as the RQ = 1).

Results

Mineralized nodules formed by MC3T3-E1 osteoblastic cell line

The mineralized nodules were micrographed at day 29 before and after Alizarin Red S staining (Fig. 2). The dye was stained at the brownish areas, the same places as seen under a light microscope, indicating the mineralized nodules formation.

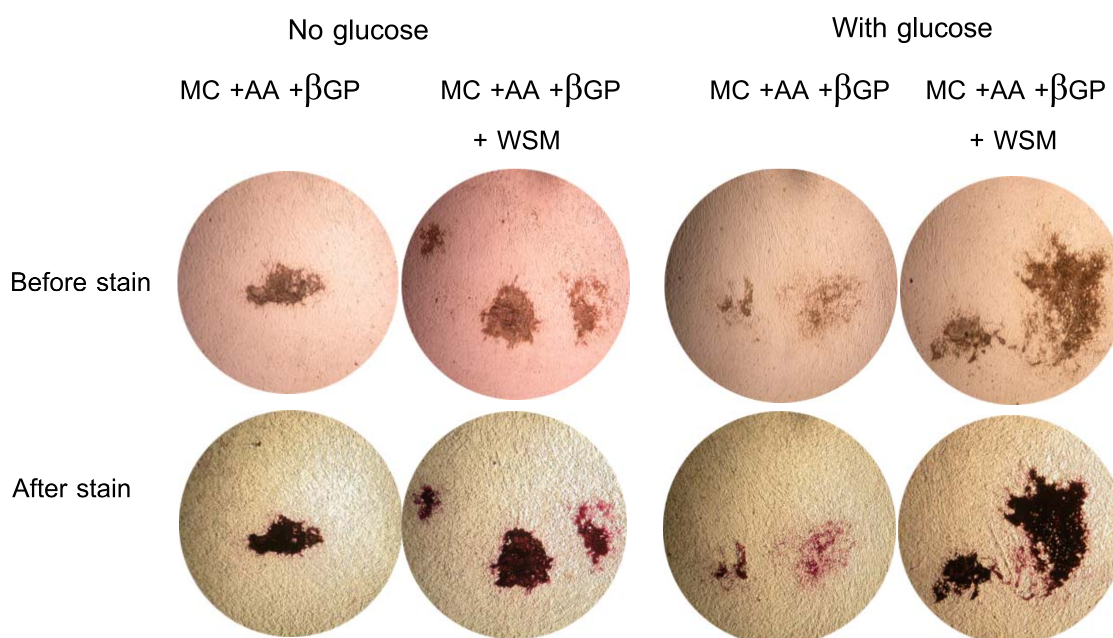

Fig. 2 Phase contrast micrographs of mineralized nodules: Before and after Alizarin Red S staining of the same culture dish (x400 magnification).

Alizarin Red S staining of mineralized nodules at different time points

The whole 35 mm culture dishes of each group were stained by Alizarin Red S and shown in Fig.3. Without extracellular glucose, the mineralized nodules were visible at day 16 after confluence. The amounts of nodules increased according to the time. With extracellular glucose, no nodules were formed at day 16 indicating the sugar delayed initiation of mineralization. However, at day 29, the groups with glucose produced more amounts of nodules than those without glucose.

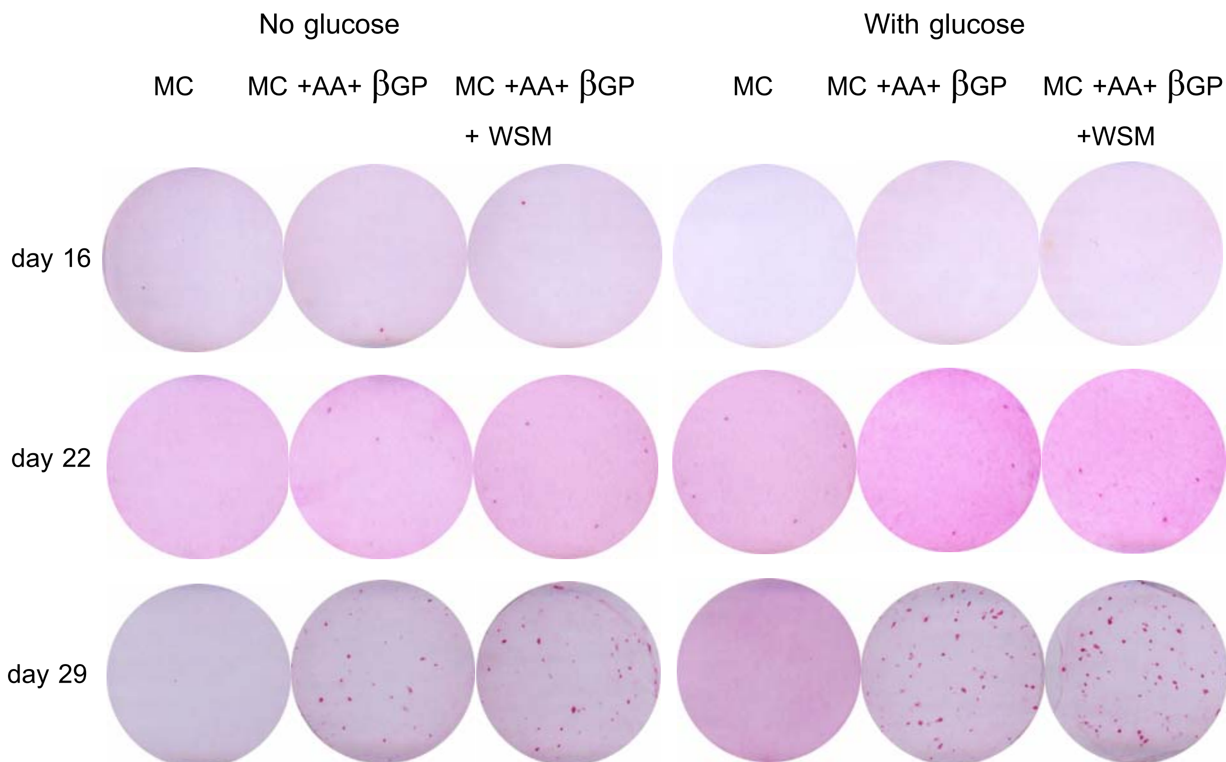

Fig. 3 Alizarin Red S staining of matrix mineralization at day 16, 22, 29

Quantification of mineralized nodules

At day 16, the mineralized areas of group 3 (MC+AA+βGP+WSM) were significantly higher than those of group 2 (MC+AA+βGP) indicating the nacre WSM enhanced initial stage of mineralization. In contrast to the groups with extracellular glucose, no mineralized nodules were formed at day 16 (Fig. 4A). At day 22, the mineralized areas of group 2 and 3 increased twice. In the presence of glucose, the mineralized nodules formed slightly higher than those without glucose (Fig. 4B). At day 29, the mineralized nodules formed by group 5 (Glu+MC+AA+βGP) and group 6 (Glu+MC+AA+βGP+WSM) were significantly higher than those without glucose (Fig. 4C). The enhanced mineralization effects of WSM could be seen in the culture without extracellular glucose (Fig. 4). The extracellular glucose delayed mineralized nodule formation (Fig. 4A); however, it increased mineralized nodules formation in the late stage of mineralization (Fig. 4C).

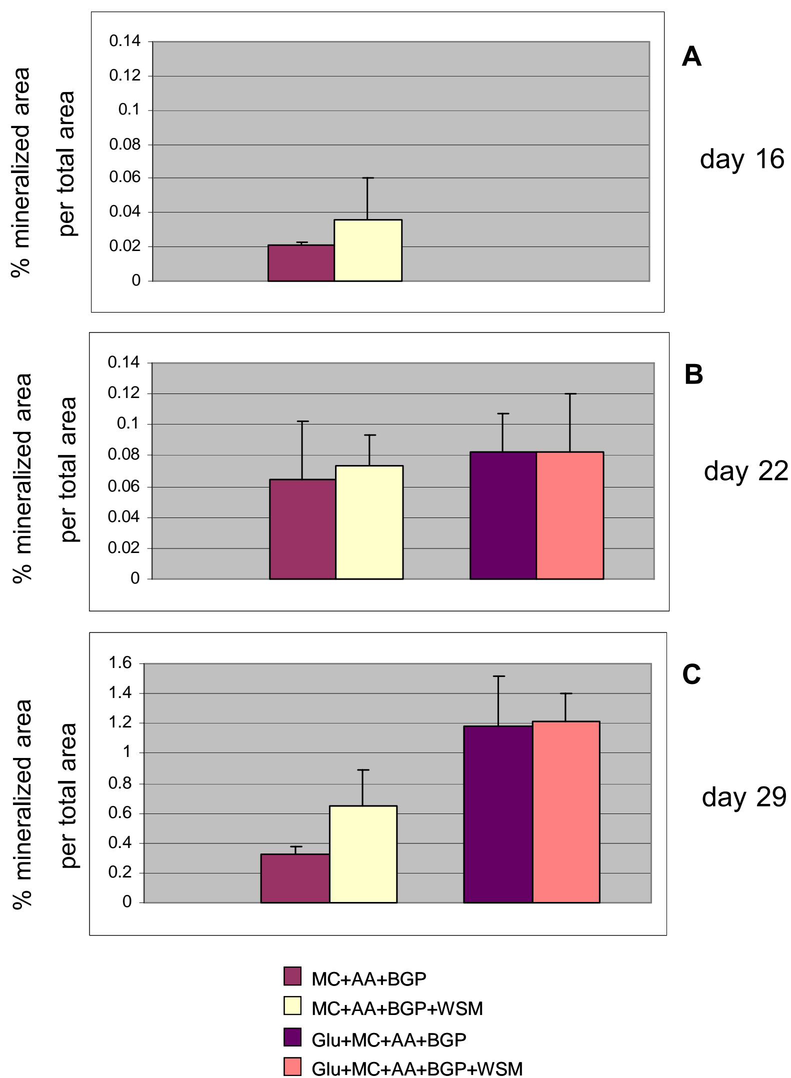

Fig. 4 Percentage of average mineralized areas per total area of 35 mm culture dish at day 16, 22, and 29 using Scion image software (Fig. 4A, 4B, 4C), respectively.

Bone matrix gene expression by relative quantitative real-time PCR

At day 7, the expression of Cbfa1 and Col1a2 were similar in pattern and no different between with or without glucose (Fig. 5A, 5B).

At day 16, the cbfa1 expression of cultures with intermittent high glucose condition was increased about three times higher than those groups without glucose (Fig. 5C). Moreover, the BSP expression of cultures with intermittent high glucose condition was dramatically increased about five times, compared to those groups without glucose (Fig. 5F).

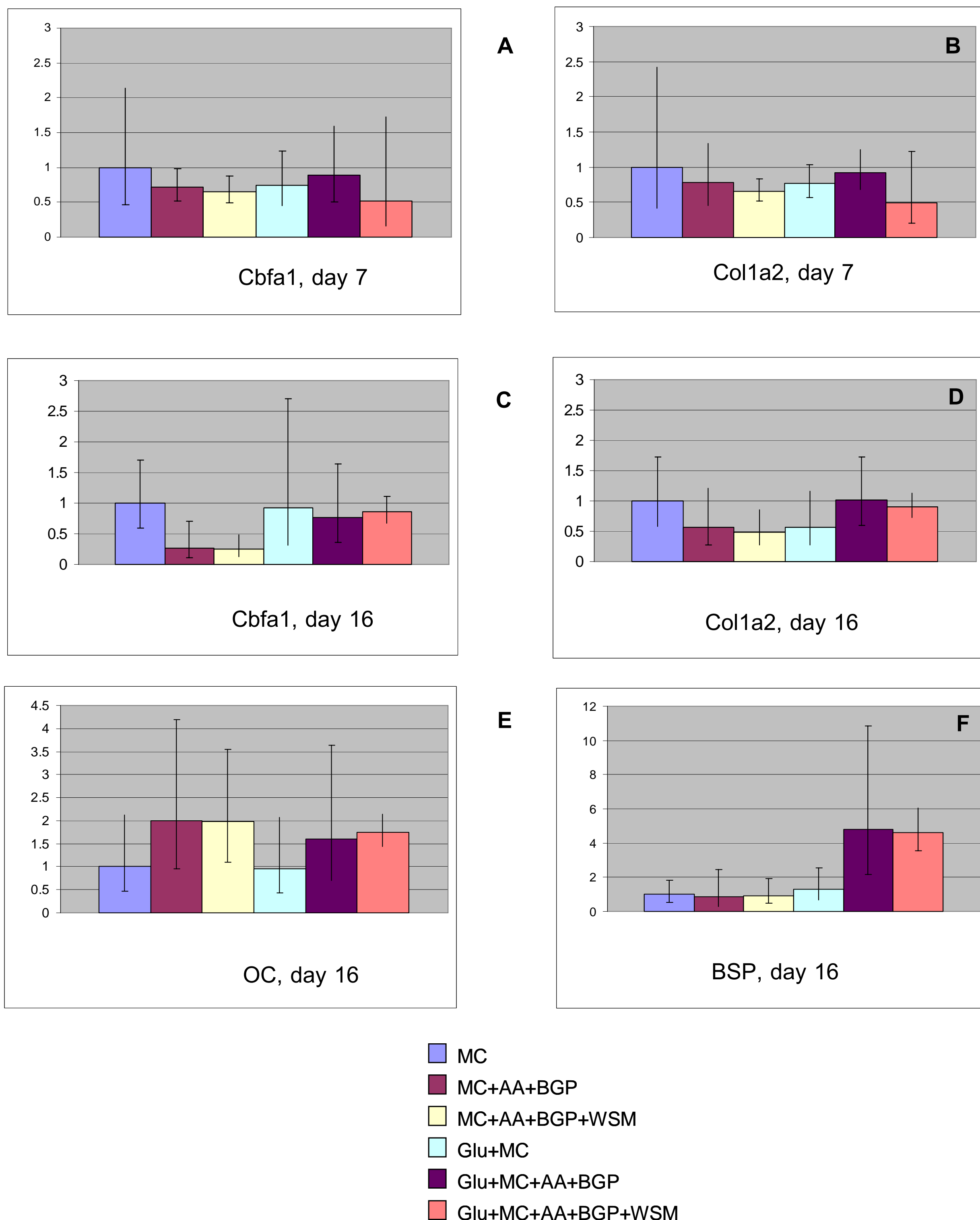

Fig. 5 Bone matrix gene expression by relative quantitative real-time PCR at day 7, 16 and day 22: A, Cbfa1 at day 7; B, Col1a2 at day 7; C, Cbfa1 at day 16; D, Col1a2 at day 16; E, Osteocalcin at day 16; F, Bone sialoprotein at day 16 (mean RQ ± SD from three 35mm culture dishes)

The Col1a2 expression of cultures with intermittent high glucose was about twice than those groups without glucose (Fig. 5D). However, the osteocalcin expression was not different among the groups with or without glucose (Fig. 5E).

At day 22, the Col1a2 expression was similar among six groups (Fig. 5G). The osteocalcin expression of culture condition without glucose were higher than that of culture condition with glucose, as shown in Fig. 5H. The BSP expression of cultures with intermittent high glucose condition was approximately 10 times higher than those without glucose (Fig. 5I).

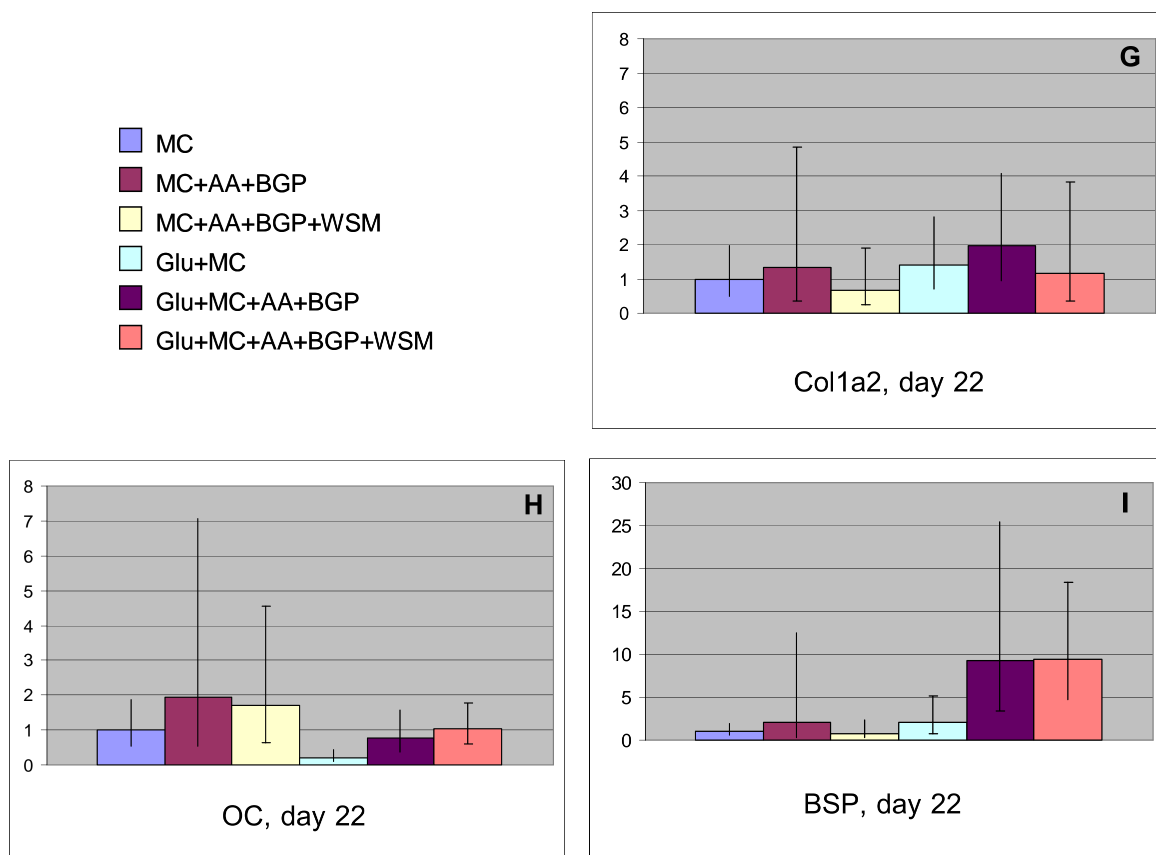

Fig. 5 (cont.) Bone matrix gene expression by relative quantitative real-time PCR: G, Col1a2 at day 22; H, Osteocalcin at day 22; I, Bone sialoprotein at day 22. (mean RQ ± SD from three 35 mm culture dishes)

Discussion

MC3T3-E1 cells is a well-characterized preosteoblastic cell line derived from mouse calvaria.[2] In the presence of 50 μg/ml AA and 2 mM inorganic phosphate, mineralizing subclone (subclone 4) of this cell line formed nodules at day 8 and expressed several osteoblastic markers including OC and BSP.[2] In the present study, MC3T3-E1 cells produced matrix mineralization at day 16, later than the original clone due to repeated passage and nonmineralizing colonies appear.

In the present study, the source of *Pinctada maxima* and the extraction of nacre WSM were different from the previous studies[3, 4] and we used only 2 mM βGP. Rousseau *et al* reported that the nacre of *Pinctada maxima* produced earlier mineralization of MC3T3-E1 cells, their study used 10 mM βGP, stained nodules at day 6, and showed only phase-contrast micrographs.[3] Mouriés *et al* showed that the nacre WSM from *Pinctada maxima* increased significantly alkaline phosphatase activity of rat bone marrow cell cultures at day 1 but the response was not dose-dependent (135, 270, 540 μg/ml WSM).[4] The nacre WSM seems to stimulate the proliferation stage of the osteoprogenitor cells.

Glucose concentrations used in the present study correspond to healthy individuals (5.5 mM D-glucose) and to poorly controlled-diabetic patient (25 mM D-glucose, 5x normal). We used the intermittent high glucose (5.5 mM D-glucose alternating with 25 mM D-glucose every 2 days) because this condition exhibited significantly different in MC3T3-E1 cells,[5] cultured rat mesangial cells[6] and cultured human tubulointerstitial cells.[7] Furthermore, the constant high glucose concentrations do not represent the conditions experienced by the cells of diabetic patients. The high glucose concentrations (15 mM or 25 mM D-glucose) have no effect on bone matrix protein as shown by previous comparisons with D-mannitol, providing equivalent osmolarity as D-glucose.[8, 9]

Clinical and *in vivo* studies suggested that diabetes has an effect on bone metabolism leading to osteopenia.[10-12] Diabetic mice showed diminished expression of osteocalcin, Cbfa1, and collagen type I.[13] *In vitro* studies in the presence of elevated extracellular glucose levels simulating diabetes resulted in a decrease calcium uptake and down regulation of osteocalcin at day 30 of MC3T3-E1 cell culture,[9] a decrease in bone marrow stromal cells proliferation, alkaline phosphatase activity and the number of mineralized nodules formed,[14] a decrease in periodontal cell proliferation and differentiation.[15] Our results clearly demonstrate that under the intermittent high glucose condition, MC3T3-E1 cells had delayed initial mineralization, however the expression of Col1a2 and osteocalcin at day 16 were not significantly different from those of controls.

Bone sialoprotein (BSP) is a mineralized tissue-specific noncollagenous protein that is glycosylated, phosphorylated and sulfated. It is a significant component of the bone extracellular matrix and has been suggested to constitute approximately 8% of all non-collagenous proteins found in bone and cementum.[16] The temporo-spatial deposition of BSP into the extracellular matrix of bone, and the ability of BSP to bind type I collagen and to nucleate hydroxyapatite crystal formation, indicates a potential role for BSP in the initial mineralization of bone, dentin and cementum.[17, 18] BSP knockout (-/-) mice showed impairment of bone growth and mineralization, concomitantly with dramatical reduction of bone formation.[19] Moreover, BSP has been associated with mineral crystal formation in several pathologies, including breast[20] and prostate carcinomas.[21] The interesting finding of the present study was the dramtically high expression of BSP in the group with intermittent high extracellular glucose at day 16 (Fig. 5F) and day 22 (Fig. 5I) which was corresponded to an increase in mineralized nodules (Fig.3, 4C). Recent study of the overexpression of BSP in MC3T3-E1 cells demonstrated an increase in several osteoblastic markers as well as mineralized nodule formation.[22] The BSP might serve as a matrix-associated signal directly promoting osteoblast differentiation resulting in the increased matrix mineralization.

Vascular calcification is common in diabetes. Bovine vascular smooth muscles incubated with high glucose (25 mM) demonstrated an increase in bone matrix protein expression including BSP and significantly enhanced calcification in a time-dependent manner.[8] Our study showed a significant increase in mineralized nodules at day 29 under the intermittent high extracellular glucose (25 mM alternatively with 5.5 mM every 2 days) (Fig. 4C). This greater nodule formation is corresponding to an increase in cbfa1 expression at day 16 and an increase in BSP expression at day 16 and day 22, a marker of osteoblastic differentiation.

There have been no previous studies investigating the effect of nacre WSM on osteoblasts in the high extracellular glucose condition. In the present study, we have studied the effect of nacre WSM on bone matrix expression and mineralization of MC3T3-E1 cells. Our results demonstrate that nacre WSM has a trend to enhance the matrix mineralization of osteoblastic cell line (MC3T3E1) in the normal medium condition (without extracellular glucose). However, in the presence of extracellular glucose, no significant effect of WSM was found on matrix mineralization.

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