Mol Cell Biochem (2010) 340:73–80
DOI 10.1007/s11010-010-0402-0

Comparative effects of a novel plant-based calcium supplement with two common calcium salts on proliferation and mineralization in human osteoblast cells

Ram Sudheer Adluri · Lijun Zhan · Manashi Bagchi · Nilanjana Maulik · Gautam Maulik

Received: 23 November 2009 / Accepted: 3 February 2010 / Published online: 7 March 2010

Abstract Calcium is an essential mineral to support bone health and serves as a major therapeutic intervention to prevent and delay the incidence of osteoporosis. Many individuals do not obtain the optimum amount of calcium from diets and depend on bioavailable calcium supplements. The present study was conducted to examine the effect of a novel plant-based calcium supplement, derived from marine algae, and contains high levels of calcium, magnesium, and other bone supporting minerals [commercially known as AlgaeCal (AC)], on proliferation, mineralization, and oxidative stress in cultured human osteoblast cells, and compared with inorganic calcium carbonate and calcium citrate salts. Cultured human fetal osteoblast cells (hFOB 1.19) were treated with AC (0.5 mg/ml, fixed by MTT assay), calcium carbonate, or calcium citrate. These cells were harvested after 4 days of treatment for ALP activity, PCNA expression, and DNA synthesis, and 2 days for Ca^{2+} deposition in the presence and absence of vitamin D3 (5 nM). The ability of AC to reduce H_2O_2 (0.3 mM)-induced oxidative stress was assessed after 24 h of treatment. ALP activity was significantly increased with AC treatment when compared to control, calcium carbonate, or calcium citrate (4.0-, 2.0-, and 2.5-fold, respectively). PCNA expression (immunocytochemical analysis), DNA synthesis (4.0-, 3.0-, and 4.0-fold, respectively), and Ca^{2+} deposition (2.0-, 1.0-, and 4.0-fold, respectively) were significantly increased in AC-treated cells when compared with control, calcium carbonate, or calcium citrate treatment. These markers were further enhanced following additional supplementation of vitamin D3 in the AC-treated group cells. AC treatment significantly reduced the H_2O_2-induced oxidative stress when compared to calcium carbonate or calcium citrate (1.5- and 1.4-fold, respectively). These findings suggest that AC may serve as a superior calcium supplement as compared to other calcium salts tested in the present study. Hence, AC may be developed as a novel anti-osteoporotic supplement in the near future.

Keywords Calcium supplement · Osteoporosis · Mineralization · Proliferation · Oxidative stress · DNA synthesis

R. S. Adluri · L. Zhan · N. Maulik
Molecular Cardiology and Angiogenesis Laboratory, Department of Surgery, University of Connecticut Health Center, Farmington, CT, USA

M. Bagchi
NutriToday, Boston, MA, USA

G. Maulik (✉)
Department of Cancer Biology, Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA
e-mail: Gautam_Maulik@dfci.harvard.edu

Introduction

Osteoporosis is a prevalent condition that may increase with age and improper nutrition, and is characterized by decreased bone mass and micro-architectural deterioration of bone tissues with a consequent increase of bone fragility and susceptibility to fracture [1]. Various calcium supplements have been used to slow the loss of bone mineral density and reduce the fracture risk. Still, adequate calcium intake remains the cornerstone of nutritional advice to maximize skeletal and bone health. Vitamin D is another critical nutrient for optimal bone health because it maintains serum calcium levels by increasing calcium absorption efficiency. Vitamin D insufficiency is associated with increased risk of hip fractures, especially in the elderly, due

to less efficient skin synthesis and intestinal absorption, reduced sun exposure and intake [2].

AlgaeCal (AC) is a plant-sourced calcium that occurs naturally in marine algae in South American coastline, which is harvested live and cold-processed to help preserve the characteristics of its phytonutrients; AC contains 73 trace minerals, of which 28–31% is calcium and 2–4% is magnesium with a pH of approximately 9.5. AC is the world's only known plant source of calcium and it also contains other bio-available pre-digested minerals.

Osteoblast cells have a pivotal role in bone metabolism. They differentiate from mesenchymal stem cells and are responsible for the synthesis of bone matrix and bone mineralization, synthesis of growth factors and hormones, and also for the regulation of osteoclastogenesis and bone resorption [3]. Osteoporosis patients have an increased risk of fractures because of the low bone mineral density and altered bone micro-architecture which are characteristic features of the disease and this may be due to decreased proliferation and mineralization of osteoblast cells [4].

Oxidative stress regulates cellular functions in multiple pathological conditions, including bone formation by osteoblast cells. Oxidative stress, resulting from excessive levels of reactive oxygen species (ROS), represents a major cause of cellular damage and death in a plethora of pathological conditions including osteoporosis, in which there are markedly increased blood levels of oxidative stress markers [5]. Osteoblast cells can produce antioxidants such as glutathione peroxidase to protect themselves against ROS, while osteoclast-generated superoxide contributes to bone degradation [6]. In ovariectomized rats, a popular model of postmenopausal osteoporosis, increased levels of lipid peroxidation and hydrogen peroxide and decreased levels of enzymatic antioxidants were demonstrated in tissue homogenates from the femora [7].

Since new bone formation is primarily a function of the osteoblast cells, agents that act by either increasing the proliferation and mineralization of the osteoblasts and/or decreasing the oxidative stress can enhance the bone formation. Therefore, the present study was conducted to test the effect of AC on proliferation, mineralization, and oxidative stress of cultured human osteoblast (hFOB 1.19) cells. The effect of AC was also compared with other two inorganic calcium sources, calcium carbonate or calcium citrate.

Materials and methods

Materials

AlgaeCal® (AC, also known as DN0361) was provided by M/s. AlgaeCal, Vancouver, Canada. Dulbecco's Modified minimal Essential Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY). Calcium carbonate, calcium citrate, and vitamin D3 were procured from M/s. Sigma Chemicals (St. Louis, MO, USA). All other reagents were of the highest commercial grade available and purchased from Sigma Chemicals (St. Louis, MO, USA).

Cell line and procedures

Human fetal osteoblast cells (hFOB 1.19-ATCC number: CRL-11372) were seeded at a density of 10,000 cells cm^{-2} in a mixture of DMEM and Ham F12 medium (1:1 ratio) supplemented with 10% FBS and 0.3 mg/ml G408. Osteoblast cells were cultured in a humidified atmosphere of 5% CO_2 at 33.5°C for cell attachment and proliferation and at 39.5°C for other assays. These alterations in temperature were required because hFOB 1.19 cells were conditionally immortalized with a gene encoding a temperature-sensitive mutant (tsA58) of SV40 large T antigen [8].

AC, calcium carbonate, calcium citrate, and vitamin D3 preparations

The content of known bone supporting minerals in AC is given in Table 1. AC, calcium carbonate and calcium citrate was dissolved in HCl:H_2O mixture at the ratio of 13:2. The stock solution of vitamin D3 was dissolved in ethyl alcohol as per the manufacturer's instructions and directly added in the medium to achieve its final concentration. The optimal dose of AC was found to be 0.5 mg/ml. The same dose was used for further experiments in the present study. The amount of calcium present in 0.5 mg of AC is 0.15 mg. Hence, this particular dose of 0.15 mg/ml of calcium from calcium carbonate and calcium citrate was used as the effective dose in further experiments. Finally, the pH was maintained same in all the preparations.

Dose determination of calcium supplements by MTT assay

Cultured osteoblast cells, at 90% confluence, were treated with different concentrations (0.1, 0.25, 0.5, and 1 mg/ml) of AC for 4 days. After 4 days, cells were washed with warm RPMI-1640 without phenol red, and then MTT (0.5 mg/ml) reagent was added into the wells and incubated for 1 h at 37°C. Then 100 µl of DMSO was added to each well and mixed well. Absorbance of the converted dye was measured at OD of 570 nm [9].

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined by enzymatic assay. Osteoblast cells, at 90% confluence,

Table 1 The content of known bone supporting minerals in 2400 mg of AlgaeCal

S. No.	Mineral	AlgaeCal average PPM	Average (%)	Amount in 2400 mg AlgaeCal (mg)	Ave. found in daily diet or dietary reference intake
1	Boron	60.2	0.0066	0.1584	0.5–1 mg[a]
2	Calcium	302200.0	30.220	725.28	1300 mg[b]
3	Copper	154.997	0.0155	0.372	0.09 mg[b]
4	Phosphorous	1021.75	0.1022	2.453	1250 mg[b]
5	Potassium	891.25	0.0891	2.138	4700 mg[b]
6	Magnesium	45040.0	4.504	108.10	420 mg[b]
7	Manganese	86.35	0.0086	0.0206	2.3 mg[b]
8	Nickel	0.224	0.000022	0.528	<100 mcg[a]
9	Selenium	0.95	0.000095	0.0023	0.055 mg[b]
10	Silica/silicon	3509.25	0.3509	8.422	5–20 mg[a]
11	Strontium	2482.5	0.2483	5.959	2 mg[c]
12	Vanadium	79.25	0.0079	189.6	10 mcg[a]
13	Zinc	5.12	0.0005	0.012	11 mg[b]

[a] See Nielsen [35]

[b] *DRI* dietary reference intake set by US Food and Nutrition Board's Institute of Medicine

[c] See Vaaro et al. [36]

treated with 0.5 mg/ml of AC, 0.15 mg/ml of calcium from calcium carbonate and calcium citrate for 4 days. After treatment, cells were rinsed with PBS, then lysed into 0.6 ml of buffer containing 10 mM Tris–HCL pH 7.5, 0.5 mM MgCl2 and 0.1% Triton X-100. Cell lysate was centrifuged at 2,000×*g* and the soluble fraction was used for enzyme assay. 50 µl of sample volumes were added to 125 µl glycine buffer (25 mM, pH 9.4), containing 2 mM $MgCl_2$ and 5 mM *p*-nitrophenylphosphate (*p*NPP), and incubated at 37°C for 50 min in a water bath. The enzymatic reaction was stopped by addition of 125 µl 1 M NaOH. The final product (*p*-nitrophenol) was quantified at 405 nm in ELx808 absorbance microplate reader, BioTek, Winooski, VT, USA. The results were normalized by the amount of cells and by specific activity (nmol *p*-nitrophenol/min/mg/ of protein). Total protein content was determined by the BCA method in aliquots of the same samples and calculated in comparison with series of bovine albumin serum as internal standards. Cultures from four independent experiments were analyzed [10].

DNA synthesis assay

The cultured osteoblast cells, at 90% confluence, were treated with AC, calcium carbonate, and calcium citrate with and without vitamin D3 for 4 days. After treatment, the cells were washed with PBS, and then added 1 µCi/ml of [^{3}H] thymidine in the serum-free medium into the cell for 4 h at 37°C. Then the cells were rinsed with 2× PBS, and then extracted twice with 10% TCA and lysed in 0.5 N NaOH. Liquid scintillation counting was performed to measure radioactivity in the lysates by using Beckman LS 6500 Multipurpose Scintillation counter, USA [11].

Immunocytochemistry for proliferating cell nuclear antigen (PCNA) expression

In order to evaluate proliferative activity, immunohistochemistry for PCNA was performed after 4 days treatment of AC, calcium carbonate, and calcium citrate. The experiment was done by following the instructions of PCNA staining kit (Invitrogen, Camerillo, CA, USA, #93-1143). Briefly, the osteoblast cells, at 90% confluence in immunocyto chambers, were incubated for 2 h at room temperature with anti-PCNA monoclonal antibody, and the labeled polymer method was applied following the manufacturer's protocol. Immunoreactivity was visualized with diaminobenzidine hydrochloride, followed by counterstaining with Meyer's hematoxylin.

Mineralization/calcium deposition assay

The osteoblast cells were treated, at 90% confluence, with culture medium containing AC, calcium carbonate, and calcium citrate with and without vitamin D3. After 2 days treatment, the cells were washed with PBS and they were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min and shaken gently. In

order to quantify the bound dye, the stain was solubilized with 10% cetyl pyridinium chloride by shaking for 15 min. The absorbance of the solubilized stain was measured at 561 nm [12].

MDA assay

After 24 h of treatment with compounds in the presence and absence of H_2O_2 (0.3 mM) [13], the cells were washed with cold PBS, scraped, and homogenized in lysis buffer. 200 µl of cell lysate was used for measuring the MDA levels by following the manufacturer's instructions. In brief, the binding of thiobarbituric acid to malondialdehyde-bis-(dimethylacetal) 1,1,3,3-tetramethoxypropan (MDA) formed during lipid peroxidation results in a chromogenic complex, which was measured at 586 nm by using DU 800 UV-VIS spectrophotometer, Beckman Coulter, Brea, CA, USA. The BIOXYTECH MDA-586 kit (OXISResearch^{TM}, CA, USA) was used to determine lipid peroxidation, which increases as a result of oxidative stress. In this study, MDA standard was used to construct a standard curve.

Statistical analysis

Data were expressed as the mean ± SEM of 3–4 independent experiments. Statistical comparisons of the results were made using one-way analysis of variance (ANOVA). Significant differences ($P \leq 0.05$) between the means of control and test group were analyzed by the Neuman–Keuls multiple comparison test.

Results

Optimum dose fixation of AC by MTT assay

In order to establish the optimum dosage, we treated the cells for 4 days at various concentrations of 0.1, 0.25, 0.5, and 1 mg/ml of AC. As it is shown in Fig. 1, the effective dose of AC is found to be 0.5 mg/ml. We found that 0.5 mg/ml showed significantly better cell survivability by MTT assay at different time points including 4 days treatment. Since 4 days is the maximum time point we have considered for other experiments related to the study, we have provided only 4 days data. The same dose was used for further experiments in the present study. The amount of calcium (the most essential mineral for bone growth) present in 0.5 mg of AC is 0.15 mg. Hence, this particular dose of 0.15 mg/ml of calcium from calcium carbonate and calcium citrate was used as the effective dose in further experiments.

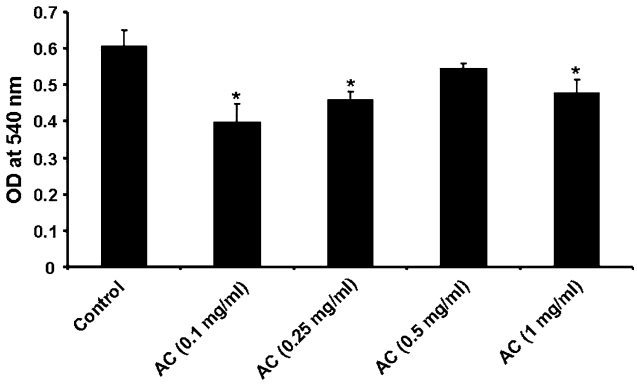

Fig. 1 Optimum dose fixation of AlgaeCal. Graph represents the optical density at 540 nm by MTT assay in AC-treated human osteoblasts at different concentrations (0.1, 0.25, 0.5, and 1 mg/ml) after 4 days of treatment. All values are expressed as mean + SEM ($n = 4$). * $P \leq 0.05$; control vs. AC different doses; *AC* AlgaeCal

Effect of AC on ALP activity with the comparison of calcium carbonate and calcium citrate

Alkaline phosphatase is an enzyme attached to the cell membrane of the osteoblasts where it increases inorganic phosphate concentration in the mineralization of extracellular vessels, favoring the precipitation of calcium phosphate, the main component of the mineral phase of bones [14]. It is also known to be involved in bone mineralization. The activity of ALP, examined after 4 days treatment, was found to significantly increase in AC-treated osteoblasts when compared to control (761 ± 59.4 vs. 189.5 ± 6.1 nmol/mg protein, $P \leq 0.05$) cells, respectively. Moreover, the effect of AC on ALP activity was significantly higher when compared with calcium carbonate (761 ± 59.4 vs. 372.2 ± 15.7 nmol/mg protein, $P \leq 0.05$) and calcium citrate (761 ± 59.4 vs. 302.2 ± 47.8 nmol/mg protein, $P \leq 0.05$), respectively, Fig. 2.

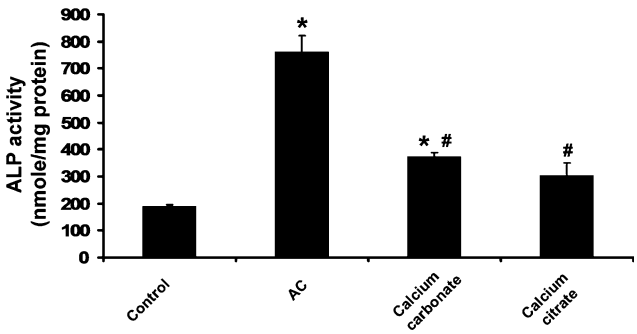

Fig. 2 Effect of AC, calcium carbonate, and calcium citrate on ALP activity of human osteoblasts after 4 days of treatment. All values are mean + SEM ($n = 4$). * $P \leq 0.05$; AC vs. control, $^{\#}$ $P \leq 0.05$; AC vs. calcium carbonate and calcium citrate; *AC* AlgaeCal

Effect of AC on PCNA expression as compared to calcium carbonate and calcium citrate

The PCNA is a 36-kDa molecular weight protein also known as cyclin. The protein has also been identified as the polymerase-associated protein and is synthesized in early G1 and S phases of the cell cycle [15]. In early S phase, PCNA has a very granular distribution and is absent from the nucleoli. At late S phase, PCNA is prominent in the nucleoli. Hence, PCNA can be used as marker for DNA synthesis and cell proliferation [15].

The expression of PCNA in AC- and calcium citrate-treated cells was considerably higher (as shown in Fig. 3) than the control- and calcium carbonate-treated cells. Addition of vitamin D3 to the AC-treated cells showed increased expression of PCNA compared to calcium carbonate + vitamin D3-treated cells, suggesting the possible role of AC as an effective calcium supplement and its absorption is high in the presence of vitamin D3.

Effect of AC on DNA synthesis with the comparison of calcium carbonate and calcium citrate

[3H] thymidine incorporation into DNA is the most commonly used method for assessing DNA synthesis and, thereby, cellular proliferation [11]. As depicted in Fig. 4, the counts were significantly more in AC-treated cells when compared to the control (210.6 ± 23.4 vs. 51 ± 14.7 cpm, $P \leq 0.05$), calcium carbonate (210.6 ± 23.4 vs. 62.8 ± 11.6 cpm, $P \leq 0.05$), and calcium citrate (210.6 ± 23.4 vs. 45 ± 9.9 cpm, $P \leq 0.05$), suggesting a possible role of AC in DNA synthesis and cell proliferation. Incubation of cells with both vitamin D3 and AC resulted in more synthesis of DNA when compared to calcium carbonate + vitamin D3 (388.9 ± 19.6 vs. 278.8 ± 74.31 cpm, $P \leq 0.05$) and calcium citrate + vitamin D3 (388.9 ± 19.6 vs. 164.2 ± 11.32 cpm, $P \leq 0.05$), indicating that AC can be absorbed more than other calcium sources in the presence of vitamin D3. This might indicate a superior bioavailability of AC over other the two calcium supplements.

Effect of AC on calcium deposition or mineralization as compared to calcium carbonate and calcium citrate

After 2 days of treatment, calcium deposition (i.e. mineralization) was found to be increased in AC-treated cells

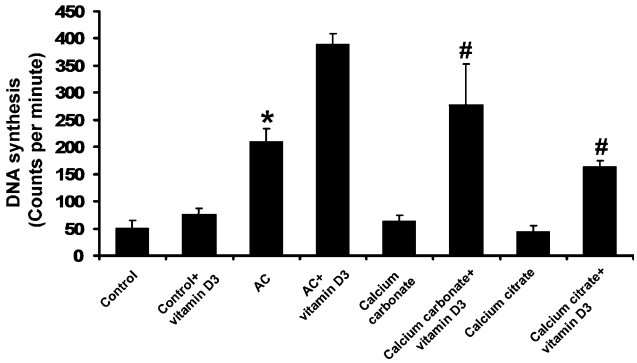

Fig. 4 Effect of AC, calcium carbonate, and calcium citrate on DNA synthesis in the presence and absence of vitamin D3. The human fetal osteoblastic hFOB 1.19 cells were cultured in the presence of AC, calcium carbonate, and calcium citrate, and/or vitamin D3 for 4 days. All values are mean + SEM ($n = 4$). * $P \leq 0.05$; control vs. AC, calcium carbonate, and calcium citrate, # $P \leq 0.05$; AC + vitamin D3 vs. calcium carbonate + vitamin D3 and calcium citrate + vitamin D3; *AC* AlgaeCal

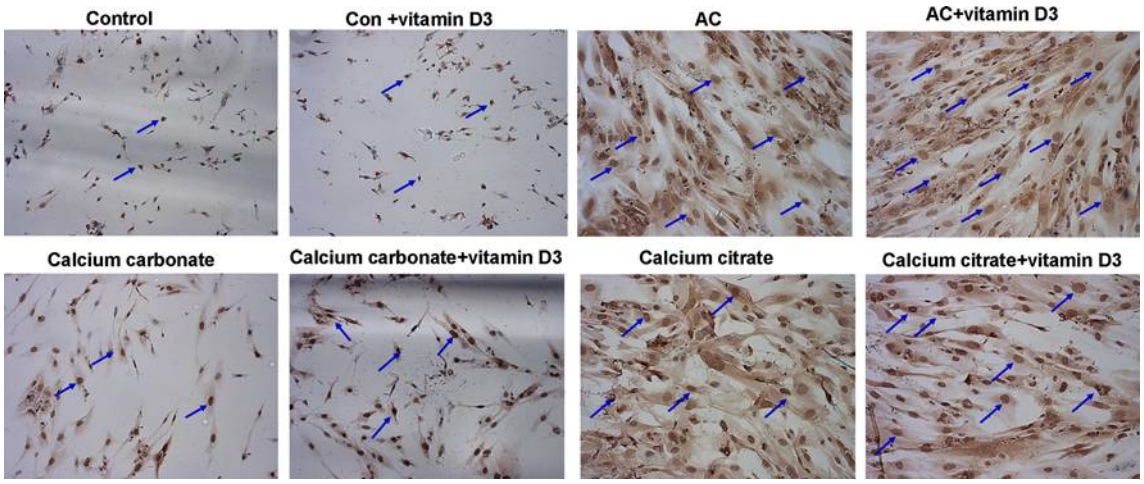

Fig. 3 Representative micrographs of immunocytochemical analysis for PCNA expression. hFOB 1.19 cells were cultured in the presence of AC, calcium carbonate, and calcium citrate with and without of vitamin D3 for 4 days ($n = 3$). Arrow represents the nuclear expression of PCNA in dividing hFOB 1.19 cells. *AC* AlgaeCal, *PCNA* proliferating cell nuclear antigen

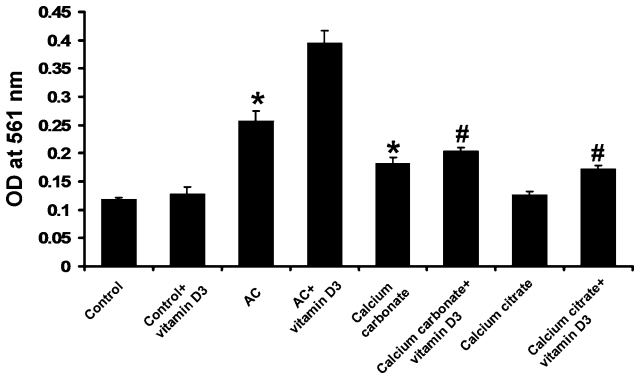

Fig. 5 Effect of AC, calcium carbonate, and calcium citrate on Ca^{2+} deposition in the presence and absence of vitamin D3. The osteoblasts were cultured in the presence of AC, calcium carbonate, and calcium citrate, and/or vitamin D3 for 2 days. All values are mean + SEM ($n = 3$). * $P \leq 0.05$; control vs. AC, calcium carbonate, and calcium citrate, # $P \leq 0.05$; AC + vitamin D3 vs. calcium carbonate + vitamin D3 and calcium citrate + vitamin D3. *AC* AlgaeCal

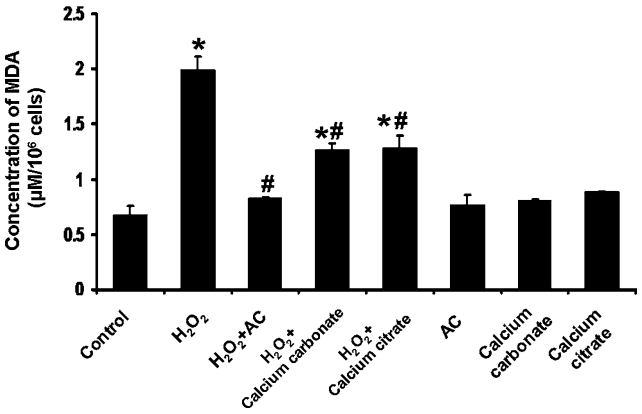

Fig. 6 Effect of AC, calcium carbonate, and calcium citrate on MDA levels against H_2O_2-induced oxidative stress. The osteoblasts were cultured in the presence of AC, calcium carbonate, and calcium citrate, and/or H_2O_2 for 24 h. All values are mean + SEM ($n = 3$). * $P \leq 0.05$; control vs. H_2O_2, H_2O_2 + AC, H_2O_2 + calcium carbonate and H_2O_2 + calcium citrate, # $P \leq 0.05$; H_2O_2 vs. H_2O_2 + AC, H_2O_2 + calcium carbonate and H_2O_2 + calcium citrate. *AC* AlgaeCal, *H_2O_2* hydrogen peroxide

compared to the control (0.2580 ± 0.016 vs. 0.1190 ± 0.0025 OD at 561 nm, $P \leq 0.05$), calcium carbonate (0.2580 ± 0.016 vs. 0.1810 ± 0.0115 OD at 561 nm, $P \leq 0.05$), and calcium citrate (0.2580 ± 0.016 vs 0.1267 ± 0.0054 OD at 561 nm, $P \leq 0.05$) treated cells, which is shown in Fig. 5. Moreover, the mineralization capacity of AC was found to be higher in the presence of vitamin D3 as compared to calcium carbonate + vitamin D3 (0.395 ± 0.0216 vs. 0.2037 ± 0.0058 OD at 561 nm, $P \leq 0.05$) and calcium citrate + vitamin D3 (0.395 ± 0.0216 vs. 0.1717 ± 0.0071 OD at 561 nm, $P \leq 0.05$).

Effect of AC on H_2O_2-induced oxidative stress as compared to calcium carbonate and calcium citrate

In order to determine the effect of AC on H_2O_2-induced oxidative damage to osteoblastic hFOB 1.19 cells, lipid peroxide (malondialdehyde; MDA) levels were assessed. As shown in Fig. 6, 0.3 mM H_2O_2 treatment increased the MDA levels more than control osteoblasts (1.992 ± 0.116 vs. 0.6768 ± 0.084 μM, $P \leq 0.05$). These results suggest that H_2O_2-enhanced ROS generation can damage lipids in osteoblast cells. However, levels of MDA in H_2O_2 + AC-treated osteoblast cells were decreased compared to only H_2O_2-treated cells (1.992 ± 0.116 vs. 0.8295 ± 0.009 μM, $P \leq 0.05$). Moreover, the anti-lipid peroxidative potential of AC was better than the other two calcium supplements, i.e., calcium carbonate (0.8295 ± 0.009 vs. 1.263 ± 0.062 μM, $P \leq 0.05$) and calcium citrate (0.8295 ± 0.009 vs. 1.289 ± 0.109 μM, $P \leq 0.05$), respectively. These results indicate that AC can also reduce oxidative damage and stress in osteoblast cells.

Discussion

In the present study, we assessed the effect of AC, a plant-based marine natural calcium supplement, on proliferation, mineralization, and oxidative stress of human fetal osteoblast cells (hFOB 1.19 cells) in vitro. ALP is a classical biomarker of osteoblast cell differentiation. Furthermore, physiologically high ALP activity is required in the early stage of the extracellular matrix mineralization, providing local phosphate ions for mineral deposition [14]. Therefore, as first step in our study, we monitored mineralization and differentiation capacity of AC by measuring the ALP activity and thereafter compared it with other calcium supplements, calcium carbonate, and calcium citrate.

The activity of ALP was significantly increased in AC-treated cells when compared with control cells as well as calcium carbonate- and calcium citrate-treated cells. The increased ALP activity in the present study by AC treatment may be due to calcium and many other minerals as magnesium, potassium, phosphorus, and zinc, which are present in significant amounts in the AC (Table 1). Recently, Heaney has suggested that many other micronutrients that are intricately involved in, and important for the health of bone, including sodium, phosphorus, magnesium, potassium, and zinc [16]. Potassium affects calcium homeostasis via urinary calcium losses. A low potassium diet causes elevated urine calcium, while the reverse is also true. It is possible that a high potassium intake can offset the bone resorption seen with a high salt diet [17]. Magnesium is found in the skeleton, and magnesium deficiency has been considered to be a risk factor for osteoporosis [18]. In a study of 70–79-year-old men and

women, magnesium intake from food and supplements was positively related to whole body BMD in white, but not black individuals [19].

Zinc is needed for osteoblastic activity, collagen synthesis, and ALP activity. Thirty percent of the zinc that is stored in the body is found in bone. Zinc is an important and abundant trace element. In addition to its presence in the mineralized component of bone, zinc is found in enzymes required for bone metabolism including ALP and carbonic anhydrase. Zinc deficiency is associated with reduced bone mass and stunted growth; however, supplementation trials in adult humans are lacking [16]. The trace mineral silicon (Si) is a readily available resource and is the next most abundant element after oxygen. Some soluble Si is found in plant-based foods (cereals, grains, some fruits, vegetables, etc.) and in unfiltered drinking water [20]. In a previous study, 4 weeks of oral Si administration (20 mg/kg body weight per day) in ovariectomized rats resulted in an increase in the bone densities of both the femoral neck and tibia [21]. Similar results were also observed in the study of Rico et al. [22] wherein ovariectomized rats were fed a Si supplement (500 mg Si/kg of feed) for 30 days.

Boron may play role in bone health through the formation of steroid hormones, and therefore, it may be involved in the prevention of calcium loss and bone demineralization. It has been shown that boron supplementation markedly reduces the urinary calcium and magnesium excretion, increases serum levels of estradiol [23], and increases calcium absorption [24], in periand postmenopausal women. In college age females, boron supplementation was shown to be related to BMD. Boron has also been related to vitamin D function by stimulating growth in vitamin D deficient animals and alleviating perturbations in mineral metabolism that are characteristic of vitamin D deficiency [25]. All these minerals are present in significant amounts in the AC.

Our results demonstrated that there was a significant increase in the number of PCNA-positive cells with AC treatment, indicating the enhancement of cell proliferation when compared to control, calcium carbonate, and calcium citrate treatment. Moreover, in the presence of vitamin D3, PCNA expression levels were high in AC-treated cells when compared with calcium carbonate- and calcium citrate-treated cells. The cell proliferation capacity of AC was further confirmed by [^{3}H] thymidine incorporation and by cell counting. The AC-treated cells showed increased amount of DNA synthesis when compared to the control, calcium carbonate, and calcium citrate. Moreover, in the presence of vitamin D3, the DNA synthesis was further increased when compared to the calcium carbonate + vitamin D3 and calcium citrate + vitamin D3 groups.

In the final step, the mineralization capacity of AC was shown by Ca^{2+} deposition assay by Alizarin red staining assay. There was a significant increase in the Ca^{2+} deposition in AC-treated cells when compared with control, calcium carbonate, and calcium citrate, respectively. The AC is known as a marine, plant-sourced mineral, hence it may be more easily absorbed than other widely used inorganic calcium salts as reported earlier [26–28] suggesting that the body is able to use less than 10% of the synthetic minerals contained in the most popular bands of multivitamins as opposed to over 80% of minerals derived from plants. Other studies have reported positive associations between fruit and vegetable consumption and BMD in people of all ages [29] including elderly adults [30, 31] and children [32]. Further, the additional minerals and vitamins that are present in the AC could have helped in the increased expression of PCNA and DNA synthesis, thereby cell proliferation and finally in the mineralization process. Our study clearly demonstrated that the effect of AC is more potent in the presence of vitamin D3 than the other two calcium supplements tested in this study. Previous studies have reported that the best recognized function of vitamin D3 is to support calcium absorption and thereby maintain blood calcium and phosphorus levels. Vitamin D3 is known to enhance calcium absorption by stimulating active transport in the gut and is absolutely necessary for effective and adequate intestinal calcium absorption [33].

The level of MDA, an oxidative stress marker, was significantly decreased when compared to H_2O_2-treated cells in the presence of AC. The anti-lipid peroxidative effect of AC was more prominent when compared to other two calcium salts tested in this study. Previous studies have demonstrated that plant-derived products like dehydrocostus lactone [13] and many other plant extracts [34] have osteoprotective effect by abolishing the oxidative stress. Since AC is a plant-derived supplement, it may contain antioxidants, which may have quenched the ROS and decreased the oxidative stress. The exact mechanism of antioxidant potential of AC is yet to be elucidated.

Taken together, this study demonstrates that natural marine calcium supplement; AC can serve as a superior and bioavailable calcium supplement than the inorganic calcium sources. The effect of AC may be due to its content of other bone supporting minerals and their influence on ALP, PCNA, and DNA synthesis; helping in the proliferation and mineralization of the osteoblast cells. Moreover, the anti-osteoporotic capacity of AC is more potent in the presence of vitamin D3. Furthermore, AC is known to significantly reduce oxidative stress in osteoblast cells as demonstrated by reduced MDA levels.

Acknowledgments This study was supported by M/s. AlgaeCal, Vancouver, Canada. We thank T. Mahesh for his help in finalizing all the figures.

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