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An *In Vitro* Study on the Effect of Five Commercial Calcium Supplements on Human Osteoblast Cell Proliferation and Ca^{2+} Mineralization

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Abstract

Calcium is an essential mineral that supports bone and joint health. It is used as a supplement, most typically as calcium carbonate, to assist in preserving bone density especially when dietary calcium intake is inadequate. In the present study we examined the effect of five varied calcium sources, three natural and two synthetic, on stimulation, proliferation and mineralization of cultured human osteoblast cells. A MTT assay using cultured human fetal osteoblast cells (hFOB 1.19) was used to determine the effective proliferative dose for salmon collagen bone calcium (0.25 mg/ml). Elemental calcium equivalence was used to select the appropriate doses for algae calcium (0.14 mg/ml), eggshell powder (0.16 mg/ml), calcium citrate (0.22 mg/ml) and calcium carbonate (0.13). Alkaline phosphatase activity, DNA synthesis rates and calcium ion deposition were evaluated after incubation under different conditions and harvesting the cells. Alkaline phosphatase activity, DNA synthesis rates and calcium ion deposition rates were all highest for the natural salmon bone collagen calcium, followed by algae calcium. The eggshell powder, calcium citrate and calcium carbonate did not exhibit significant changes from control in most of these assays. The results of these assays suggest that natural marine collagen calcium such as salmon bone collagen calcium acts as the most effective intervention on osteoblast performance and actual calcium deposition. It should be recommended as a superior supplement for improved bone and joint health function.

Keywords: Osteoporosis; Calcium supplement; Salmon; Osteoblast cells; DNA synthesis; Bone mineralization

Introduction

Osteoporosis is characterized by a decrease in bone mass and micro-architectural deterioration of bone tissues with a consequent increase in bone fragility and susceptibility to fracture [1]. It is a globally prevalent condition that rapidly advances with age, particularly in women [2]. Various calcium supplements are in use to help slow the characteristic loss of bone mineral density and reduce the risk of fracture but with limited success and some elevated co-morbidity risks [3]. In spite of these negative results, increasing calcium intake through food fortification and nutraceutical supplements remains the primary advice to maintain skeletal and bone health [4]. Probiotic supplementation has also been described to help with osteoporosis [5].

Salmon bone collagen calcium (CalGo®) is a marine-sourced, by-product made by enzymatic hydrolysis of salmon off-cuts. The hydrolysis is followed by separation of the oil, soluble protein and solids fractions that yields clean bones. These bones are powdered and dried to yield the salmon bone collagen calcium used in this trial. The freshness of the salmon offcuts and the mild extraction conditions used to produce the calcium powder are expected to make a positive impact on bone health, via the presence of natural bone protein growth factors, active trace minerals and type II collagen containing hydroxyapatite matrix.

Osteoblast cells play a pivotal role in bone metabolism. They are responsible for the synthesis of bone matrix and bone mineralization, synthesis of growth factors and hormones, and also for the regulation of osteoclast genesis and bone resorption [6]. A decrease in the proliferation and mineralization of osteoblast cells leads to low bone mineral density and altered bone micro-architecture. These symptoms are characteristic in osteoporotic patients who have an increased risk of fractures [7]. Osteoblast cells produce antioxidants such as glutathione peroxidase to protect themselves against reactive oxygen species which are a major cause of cellular damage and death in a plethora of pathological conditions, including osteoporosis [8]. Since new bone formation is primarily a function of osteoblast cells, agents that either increase the proliferation and mineralization of the osteoblasts and/or decrease their oxidative stress should enhance bone formation.

The present study was conducted to test the effect of salmon bone collagen calcium on proliferation, mineralization, and oxidative stress in cultured human osteoblast (hFOB 1.19) cells. A positive response in this assay could pave the way to the development of a moderate calcium ion content nutraceutical for use during pregnancy to help reduce the occurrence of preeclampsia, while still providing good joint health supplementation. The effect was also compared with four commercial calcium sources, calcium carbonate, calcium citrate, eggshell powder and algal calcium to understand the impact of each of the components present in the salmon bone collagen calcium.

Materials and Methods

Materials

Salmon bone collagen calcium (also known as CalGo) was provided by Hofseth Biocare AS, Aalesund, Norway. M/s. Eggshell powder was purchased from Nature-Egg LLP (Ghaziabad, India). Algae calcium (AlgaeCal Basic) was purchased from AlgaeCal Inc. Vancouver, Canada). All other reagents were of the highest commercial grade available and purchased from Millipore Sigma (St. Louis, MO, USA).

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Salmon bone collagen calcium, calcium carbonate, calcium citrate, eggshell powder and algae calcium powder specification

Calcium carbonate and calcium citrate are synthetic products with no detectable trace minerals present. The ppm content of potential bone supporting minerals in the other three naturally sourced materials - salmon bone collagen calcium, eggshell powder and algae calcium are based on reported values [9-11] and are shown below Table 1.

In addition, salmon bone collagen calcium is the only powder in this trial that contains approximately 20% w/w of un-hydrolyzed type II collagen as well as other protein growth factors that may also influence osteoblast cell proliferation.

Cell line preparation

Human fetal osteoblast cells (hFOB 1.19-ATCC number: CRL-11372) were seeded at a density of 10,000 cells cm^2 in the Millipore (D6421) mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham F12 medium (1:1 ratio) supplemented with 10% Fetal Bovine Serum (FBS). The cells were cultured in a humidified atmosphere of 5% CO_2 at 35°C.

Dose determination for use of salmon bone collagen calcium by MTT assay

Cultured osteoblast cells at 90% confluence were treated at four concentrations (0.1, 0.25, 0.5 and 1 mg/ml) of salmon bone collagen calcium for 4 days at RT. The solutions were made by dissolving the salmon bone collagen calcium in 1M HCl solution. After 4 days, cells were washed with warm RPMI-1640 and incubated for 1 h at 35°C in the presence of 0.5 mg/ml MTT reagent. After 1 h, 100 microliters of dimethyl sulfoxide was added to each well, mixed and absorbance measured at OD of 540 nm.

Alkaline phosphatase activity

Alkaline phosphatase is an excellent proxy biomarker for studying bone mineralization because it binds to osteoblast membranes and increases inorganic phosphate concentrations in extracellular vessels. It plays a critical role in the precipitation of calcium phosphate, the main component of the mineral fraction of bones [12].

Alkaline phosphatase (ALP) activity in this study was determined by an enzymatic assay (Abcam Plc., Cambridge, UK). Based on the MTT assay, osteoblast cells at 90% confluence were incubated with 0.25 mg/ml of salmon bone collagen calcium in 1M HCl and elemental calcium equivalent doses of the other test samples at 0.13 mg/ml of calcium carbonate, 0.22 mg/ml of calcium citrate, 0.16 mg/ml of eggshell powder and 0.14 mg.ml of algae calcium powder, for 4 days at room temperature (25°C). After treatment, cells were rinsed with phosphate-buffered saline (PBS), then lysed into 0.6 ml of 7.5 pH buffer containing 0.1% Triton X-100. The cell lysate was centrifuged at 4000 RPM and the soluble fraction was used for the enzyme assay. 50 ul of each sample was added to 125 ul glycine buffer (pH 9.4), containing 2 mM magnesium chloride and 5 mM p-nitrophenyl phosphate (pNPP), and incubated at 36°C for 1 h. The reaction was stopped by addition of 125 ul 1M sodium hydroxide and the released p -nitrophenol was quantified at 405 nm in a FluoDia T70 absorbance microplate reader, PTI Inc., Birmingham, NJ, USA and normalized for # of cells against the standard curve of nmol p -nitrophenol/min/mg/ of protein. Total protein content was determined by the Bicinchoninic acid (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 [13].

DNA synthesis assay

Incorporating [^{3}H] thymidine into DNA and measuring the radioactivity is a routine assay to measure rates of DNA synthesis and cellular proliferation [14].

90% confluence osteoblast cells were treated with 0.25 mg/ml of salmon bone collagen calcium, 0.13 mg/ml of calcium carbonate, 0.22 mg/ml of calcium citrate, 0.16 mg/ml of eggshell powder and 0.14 mg.ml of algae calcium powder, for 4 days at room temperature. The cells were washed with PBS, and then incubated at 37°C for 4 h in the presence of 1 ul Ci/ml [^{3}H] thymidine, rinsed with PBS and extracted twice with 10% trichloroacetic acid (TCA). Cell lysis in 0.5 N sodium hydroxide resulted in the ready sample for measuring radioactivity count using a Beckman LS 5000 Scintillation Counter, USA [15].

Mineralization/calcium deposition assay

The osteoblast cells at 90% confluence, were treated for 2 days with a culture medium and 1M HCl solutions of 0.25 mg/ml of salmon bone collagen calcium, 0.13 mg/ml of calcium carbonate, 0.22 mg/ml of calcium citrate, 0.14 mg/ml of eggshell powder and 0.16 mg/ml of algae calcium powder for 2 days. The cells were washed with PBS and they fixed with 70% ethanol for 1 h. Staining with 40 mM Alizarin Red S for 10 min and shaking gently bound the dye to the cells. The bound dye was quantified by solubilizing it in10% cetyl pyridinium chloride by shaking for 15 min.

The absorbance of the solubilized stain was measured at 561 nm against a standard curve [16].

	Salmon Bone powder (ppm)	Eggshell powder (ppm)	Algae Calcium powder (ppm)	Calcium carbonate (ppm)	Calcium citrate (ppm)
Boron	-	0.5	60	np	np
Calcium	208000	365000	302200	399900	241200
Copper	17	8	154		
Iron	24	22	-		
Magnesium	4100	4400	4500	np	np
Manganese	66	7130	86	np	np
Phosphorous	156000	910	1020	np	np
Potassium	1300	-	890	np	np
Selenium	0.3	0.2	1	np	np
Silicon	-	-	3500	np	np
Strontium	-	372	2500	np	np
Zinc	17	5	5	np	np

* '-' = not tested ; 'np' = not present.

Table 1: Content of minerals in the five samples of calcium powders in this study.

Statistical analysis

All data is expressed as the mean of 3 independent experiments. Statistical comparisons of the results were done using one-way analysis of variance (ANOVA).

Significant differences ($P<0.05$) between the means of control and test groups were analyzed by the Holm-Sidak test.

Results

Optimum dose determination in osteoblast cells for salmon bone collagen calcium using MTT assay

We established the optimum dose by treating the osteoblast cells for 4 days at concentrations of 0.1, 0.25, 0.5 and 1 mg/ml of salmon bone collagen calcium for 4 days at room temperature. As shown in Figure 1, the most effective dose of salmon bone collagen calcium is found to be 0.25 mg/ml wherein we see significantly better cell survivability. This dose has been fixed for the rest of the present study. Since the amount of elemental calcium present in 0.25 mg of salmon bone collagen calcium is 0.05 mg, the same level of elemental calcium is used in fixing the treatment doses of the other four calcium powders at; 0.13 mg/ml of calcium carbonate, 0.22 mg/ml of calcium citrate, 0.14 mg/ml of eggshell powder and 0.16 mg/ml of algae calcium powder in the further experiments.

Effect of the five calcium powders on alkaline phosphatase activity

Alkaline phosphatase levels were examined after 4 days treatment with the above doses of the five treatments. Significant increases were only noted in the salmon bone collagen calcium and algae calcium treated osteoblasts when compared to control cells. (815 ± 46, 598 ± 31 respectively versus control at 287 ± 33 nmol/mg protein, $P<0.05$)

The effect of treatment with eggshell powder, calcium carbonate and calcium citrate was not significantly different from control (332 ± 37, 299 ± 19, 333 ± 31 nmol/mg protein respectively) as shown in Figure 2 below.

Effect of the five calcium powders on DNA synthesis

Counts were measured for all five test powders in triplicate. Significantly higher values were seen for salmon bone powder and algae calcium as compared to control (232 ± 27 and 202 ± 20 cpm respectively versus 47 ± 9 cpm, $P<0.05$). Eggshell powder, calcium carbonate and calcium citrate also showed significant increases versus control (161 ± 23, 114 ± 14, 136 ± 28 cpm respectively, $P<0.05$) as shown in Figure 3 below. The increased DNA synthesis and potential osteoblast cell proliferation potential of salmon bone collagen calcium could be because of the exclusive presence of high levels of collagenic and growth inducing proteins as compared to the other calcium sources.

Effect of the five calcium powders on calcium deposition and mineralization

Calcium deposition and mineralization was found to be increased in all five powder treated cells as compared to the control. Salmon bone collagen calcium and algae treatment both showed the significant increases in calcium deposition versus control (0.20 ± 0.04, 0.21 ± 0.04 OD at 561 nm respectively versus control at 0.11 ± 0.02 OD at 561 nm, $P<0.05$) as shown in Figure 4 below. Eggshell powder, calcium carbonate and calcium citrate did not show any increase versus control.

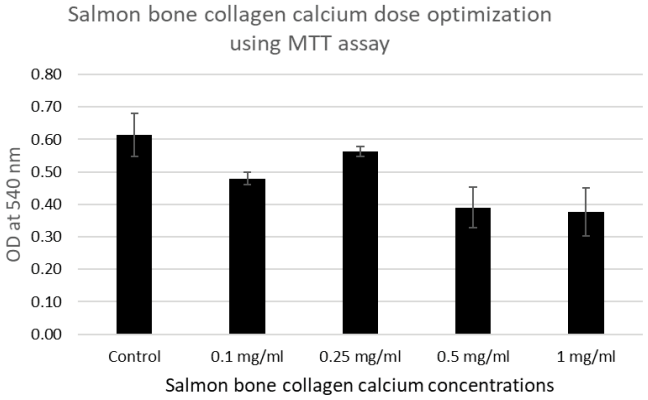
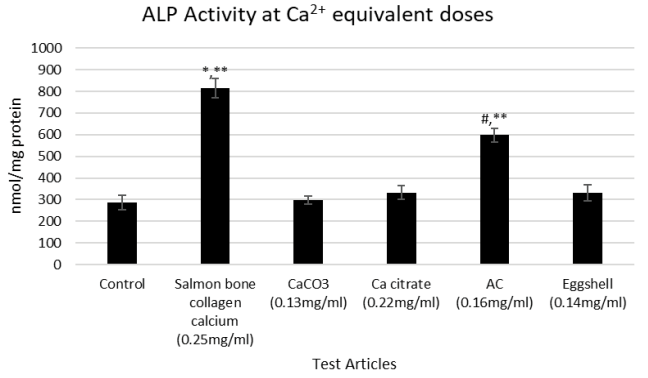
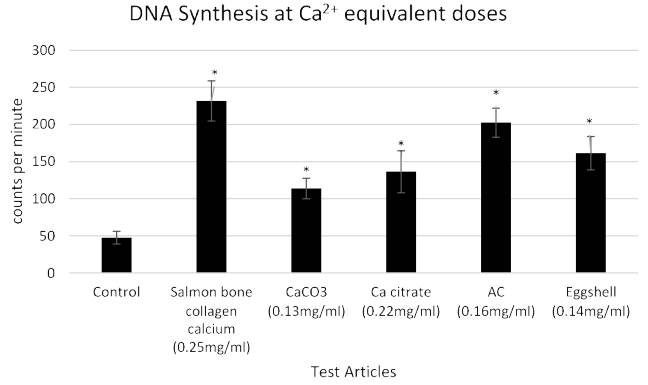


Figure 1: Optimum dose fixation of salmon bone collagen calcium by MTT assay in human osteoblasts after 4 days treatment (n=3).



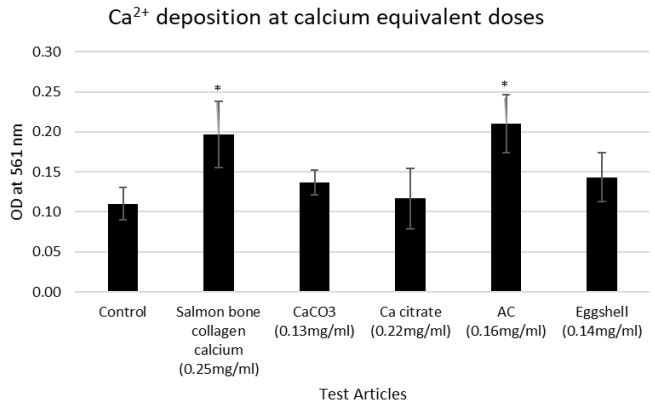
*P<0.05; salmon bone collagen calcium, algae calcium *vs.* control, # P<0.05; algae calcium *vs* control, ** P<0.05; salmon bone collagen calcium, algae calcium *vs*. eggshell powder, calcium carbonate and calcium citrate.

Figure 2: Effect of salmon bone collagen calcium, algae calcium, eggshell powder, calcium carbonate and citrate on ALP activity of human osteoblasts after 4 days of treatment (n=3).



*P<0.05; salmon bone collagen calcium, algae calcium, eggshell powder, calcium carbonate and calcium citrate vs control.

Figure 3: Effect of salmon bone collagen calcium, algae calcium, eggshell powder, calcium carbonate and citrate on DNA synthesis in human fetal osteoblast hFOB 1.19 cells (n=4).



*P <0.05; salmon bone collagen calcium and algae calcium *vs* control.

Figure 4: Effect of salmon bone collagen calcium, algae calcium, eggshell powder, calcium carbonate, and calcium citrate on Ca^{2+} deposition using osteoblasts cultured in the presence of the test articles for 2 days (n=3).

Discussion

In this present study we studied the effect of salmon bone collagen calcium on the proliferation and mineralization of human fetal osteoblast cells (hFOB 1.19 cells) *in vitro* using three assays - alkaline phosphatase levels, DNA synthesis changes and Ca^{2+} deposition. We also compared these results against four other sources of nutraceutical calcium powders.

We first determined the most appropriate assay dose for the osteoblast cells using a MTT cell proliferation assay with salmon bone collagen calcium. We used this result to match the elemental calcium equivalent doses of the other four calcium sources for the rest of the assays.

Alkaline phosphatase has been used as a biomarker of osteoblast cell differentiation and the early stages of extracellular matrix mineralization, acting as a local source of the phosphate ions needed for mineral deposition [17]. The activity of alkaline phosphatase was significantly increased in salmon bone collagen calcium, while being lower but still significant in algae calcium and eggshell powder treatments as compared to control cells as well as calcium carbonate and calcium citrate treatments. The increased alkaline phosphatase activity observed with the salmon bone collagen calcium, algae calcium and eggshell powder may be accounted for by the presence of additional minerals that are not present in the calcium carbonate and citrate powders as shown in Table 1. Low boron and potassium intake in the diet have been shown to lead to elevated urine calcium and bone demineralization [18] while supplementation has shown an increase in calcium absorption [19]. Magnesium deficiency has also been shown to be a risk factor for osteoporosis [20]. Other studies have shown that micronutrients may also play a significant role in bone and joint health [21]. Zinc is an important micronutrient with 30% of its body presence found in bone. It is a component in bone metabolism enzymes such as alkaline phosphatase and carbonic anhydrase [22]. The increased alkaline phosphatase activity may be particularly driven by the high amount of phosphorus (hydroxyapatite) which shows a proportional increase to alkaline phosphatase production, in this study.

The presence of type II collagen in the salmon bone collagen calcium powder may also play a role in increasing alkaline phosphatase production. Zinc also plays a pivotal role in collagen synthesis [23], particularly for type II collagen which is found predominantly in joint cartilage tissue [24]. Wang et al. have shown that hydroxyapatite/collagen microspheres can be used to support the growth and proliferation of osteoblast cells so as to find use as filling materials for bone defect [25]. These trace minerals and type II collagen present in natural sources of calcium such as salmon bone collagen calcium, could be playing a major role in the increased alkaline phosphatase activity observed in this study.

This increased alkaline phosphatase activity translated to an increase in DNA synthesis as confirmed by [^{3}H] thymidine incorporation for all five powders tested. The algae calcium and salmon bone collagen calcium treated cells showed the maximum increased amounts of DNA synthesis when compared to the control, calcium carbonate, calcium citrate and eggshell powder.

Our final assay directly measured the mineralization capacity of the five calcium powders using Ca^{2+} deposition using Alizarin red staining measurements. There was a significant increase in calcium deposition in the salmon bone collagen calcium and algae calcium treated cells followed by a moderate but not statistically significant increase in eggshell calcium treated cells versus no increase in control, calcium carbonate, and calcium citrate treated cells. It has been reported that damage to type II collagen in aging and osteoarthritic patients starts at the articular surface and leads to progressive cartilage degeneration [26]. Our results show it is possible that the salmon bone collagen calcium and algae calcium may help supplement this lost collagen and reduce such damage to articular surfaces, thereby assisting in joint health improvements.

It can be concluded that the additional growth factors, type II collagen and micronutrient minerals that are significantly present in the salmon bone collagen calcium and algae calcium may be contributing to the increased ALP and DNA synthesis osteoblast cell proliferation observed in this study.

Taken together, the results of this study validates previous dietary studies [27] that show that consuming small fish bones in the diet improves bone and joint health. Our study shows that natural algae calcium or fish bone supplements, such as salmon bone collagen calcium, may serve as a superior, bioavailable calcium supplement as compared to other mono-component organic or inorganic calcium supplement powders. The lower calcium ion content may play a significant role in the development of this powder specifically for use during pregnancy and preeclampsia. The improved effect is likely due to several factors: the presence of calcium in its phosphorus-rich hydroxyapatite form, the presence of bone-supporting micronutrient minerals and their influence on alkaline phosphatase activity and the presence of type II collagen that helps increase DNA synthesis and proliferation and Ca^{2+} deposition in human osteoblast cells.

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