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Different multi-nutrient formulations support calcification process in human bone cells

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Introduction

Bone is a specialized connective tissue and its formation involves many steps and stages. It is a dynamic tissue that responds to many internal and external factors and adapts to changing functional conditions. Bone is composed of various types of cells and collagenous extracellular organic matrix, which is predominantly type I collagen (85–95%) that becomes hardened by the deposition of calcium hydroxyapatite. Bone also contains various proteins and proteoglycans, which are specific to bone and dental hard connective tissues. Healthy bone metabolism depends on the proper function of different types of bone cells, such as osteoclasts which resorb (dissolve) the bone, osteoblasts that build the bone, osteocytes that help maintain bone, and lining cells that cover the surface of the bone. Osteoblasts activate bone growth and differentiate to osteocytes during bone formation. The osteocytes respond to mechanical stress and play an important role in bone remodeling.

Several bone-specific metabolic markers have been used to study bone metabolism:

- Alkaline phosphatase (ALP) is one of the most frequently used clinical markers for bone forming. It is a key enzyme synthesized by osteoblasts at early stages of bone mineralization and supports the calcification of bone matrix.

- Osteocalcin is a vitamin K-dependent protein secreted by osteoblasts. It accumulates in bone matrix and is important for binding calcium and for bone mineralization.

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- Sclerostin protein is secreted by osteocytes. High levels of sclerostin not only inhibit osteoblasts proliferation and their differentiation to osteocytes, but also induce death of osteoblasts, thus inhibiting bone formation. Sclerostin has become a popular therapeutic target in treating osteoporosis.

The relationship and effects of each marker on bone formation is outlined in Figure 1.

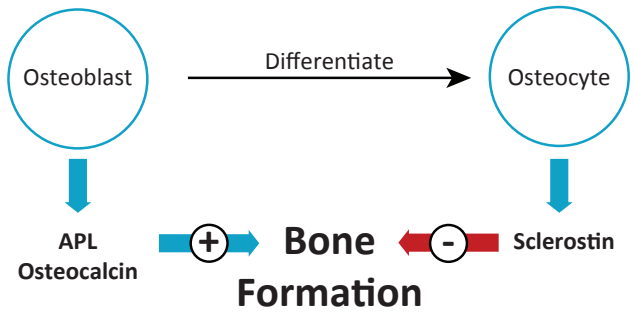

Figure 1. Significance of metabolic markers tested in the study on bone formation. (+) = promotion; (-) = inhibition.

Micronutrients regulate various biological processes and affect bone formation at different metabolic stages. Vitamin C plays a prominent role in bone health as an essential nutrient in the synthesis and structure of collagen, which forms the protein foundation of the bone on which minerals are deposited, hardening the entire structure. In addition, vitamin D and K, minerals (i.e. calcium, magnesium), and other specific nutrients regulate the bone mineralization process and metabolism at the level of cells.

Many people concerned with bone health take nutritional supplements, such as vitamin D, calcium, or vitamin K. There are also various multi-nutrient supplements combining vitamins, minerals and other nutrients that claim bone health benefits. Many of these formulas contain similar sets of ingredients; however, they differ in respect to the chemical forms and quantities used in the formulation as well as sources of raw materials. Rarely, if at all, are these formulas as a whole tested for their biological efficacy.

In our study, we investigated the effects of three multi-micronutrient formulas developed by the Dr. Rath Research Institute on specific metabolic processes in human bone-building cells. Formula A is a multivitamin/multi-nutrient composition that supports general cell metabolism in the body; Formula B contains a selection of micronutrients important for bone health; Formula C contains key essential nutrients supporting collagen formation. The ingredients in each formula can target different cellular functions; therefore, we tested their effects on bone building both individually and combined.

Material and Methods

Cell Culture and treatment

Human Osteoblast cells (HOb) were purchased from ATCC. The cells were cultured in Dulbecco Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin. Incubation was conducted at 5% CO_2 at 37°C. The medium was changed every 2–3 days. Micronutrient Formulas A, B, and C were provided by Dr. Rath International Inc. (San Jose, CA). As for all experiments, cells were seeded in 96 well plates (1 x 10^5 cells/well) in 10% FBS/DMEM. When cells reached confluence at 37°C, they were treated with each test formula at a concentration of 0.125 mpdd.

Preparation of test formulas

All nutritional supplements were treated identically in accordance with the protocol recommended by the United States Pharmacopeia. Three recommended daily doses of each formula were crushed using ceramic pestle and mortar to powder. The powder was then dissolved in 900 ml of 0.1N hydrochloric acid and incubated for 1 hour at 37°C in a shaking incubator set with rotation speed of 75 rpm. Resulting solutions were filter-sterilized using 0.2 micrometer pore size filters, aliquoted and kept frozen at -20°C until used. Amounts of samples applied in the experiments are expressed as number of millionth parts of recommended daily dose of the respective supplement.

Alkaline phosphatase (ALP) assay

ALP assay was performed by using Alkaline Phosphatase Activity Colorimetric Assay Kit (BioVision, Milpitas, CA). Briefly, after 48 hours exposure to the test formula as described above, cells were lysed with the assay buffer. Subsequently, 5 mM p-nitrophenyl phosphate (pNPP)—a substrate for alkaline phosphatase—was added into the experimental wells for 1 hour, followed by stop solution. The absorbance was measured at 450 nm within 5 minutes using a microplate reader.

Sclerostin and Osteocalcin assays

Cells were treated with each test formula for 2 weeks as described above to allow osteoblasts to differentiate to osteocytes. Both medium and formula treatments were replaced every 3 days. After 2 weeks, cells were washed 3 times with PBS and incubated with 3% formaldehyde/0.1% Trition in PBS for 1 hour at 4°C. Human SOST/Sclerostin antibody (R&D Systems MAB1406-SP) and human/rat osteocalcin antibody (R&D Systems MAB1419-SP) were used for sclerostin assay and osteocalcin assay, respectively. ELISA was run by using antibodies mentioned above at 1:1k dilution for 2 hours at room temperature. Then anti-mouse HRP (Pierce) at 1:1k dilution was added for 1 hour at room temperature. Subsequently, cells were washed 3 times with 0.1% BSA/PBS. TMB substrate solution (Rockland) was added for peroxidase reaction. After 20 minutes, the absorbance was measured at 650 nm using a microplate reader.

Statistical analysis

Mean and standard deviations of the data were calculated using Microsoft Excel. Significant differences between control and each formula treatment were determined by student's t-tests at a significance level of 0.05.

Results

Characteristic of micronutrient formulas tested in the study

Nutrient composition of Formulas A, B and C is presented in Table 1.

Table 1. Nutrient composition of Formulas A, B and C

Formula A		Formula B	Formula C
Vitamin C	Molybdenum	Vitamin C	Vitamin C
Vitamin A	Biotin	Vitamin A	L-lysine
Vitamin E	L-proline	Vitamin E	L-proline
Vitamin D3	L-lysine	Vitamin D3	
Vitamin B	L-carnitine	Vitamin K2	
Folic acid	L-arginine	Folic acid	
Calcium	L-cysteine	Calcium	
Magnesium	Coenzyme Q10	Magnesium	
Potassium	Phosphorus	Potassium	
Zinc	Pycnogenol	Zinc	
Manganese	Citrus bioflavonoids	Manganese	
Selenium	Natural carotenoids	Boron	
Copper		Iodine	
Chromium		Silica	
Inositol		Natural carotenoids	

Formula A provides basic nutrients needed by all cells in the body to support cell growth, normal energy metabolism, collagen synthesis, oxidative stress protection and many other functions. It comprises over 30 natural components acting synergistically, such as vitamins, amino acids, minerals, and other essential nutrients.

Formula B was designed to support bone cell metabolism. It contains some nutrients that are also present in Formula A and C, such as vitamin C, which is essential for collagen synthesis in bone cells. Formula B also contains vitamin K, vitamin D, and important minerals for bone building, such as calcium, magnesium, and boron. Magnesium and boron assist the absorption and metabolism of calcium required for bone strength and density.

Formula C contains key essential nutrients for collagen formation: vitamin C, lysine, and proline. Vitamin C regulates collagen synthesis and its hydroxylation, assuring proper assembly of a triple helix collagen structure. Lysine and proline—which compose about 2/3rd of collagen amino acids—form collagen fibrils as the major protein in connective tissues in the body. Because humans do not produce vitamin C and lysine innately, deficiencies of these essential nutrients are likely to occur, which have negative consequences on collagen synthesis in the body, including bone collagen. Humans produce proline, but to a limited degree.

Metabolic effects of two multinutrient formulas on bone health: Formula A and Formula B

The results in Figure 2 show that Formulas A and B have positive effects on all test markers relevant for bone health. As such, exposure of bone cells to Formulas A and B resulted in higher alkaline phosphatase (ALP) activity by 15% and 43% respectively, compared to control (cells not exposed to micronutrient treatment) (Fig. 2-I). Increased activity of ALP has been positively associated with mineralization processes in bones. Figure 2-II shows that bone-building cells exposed

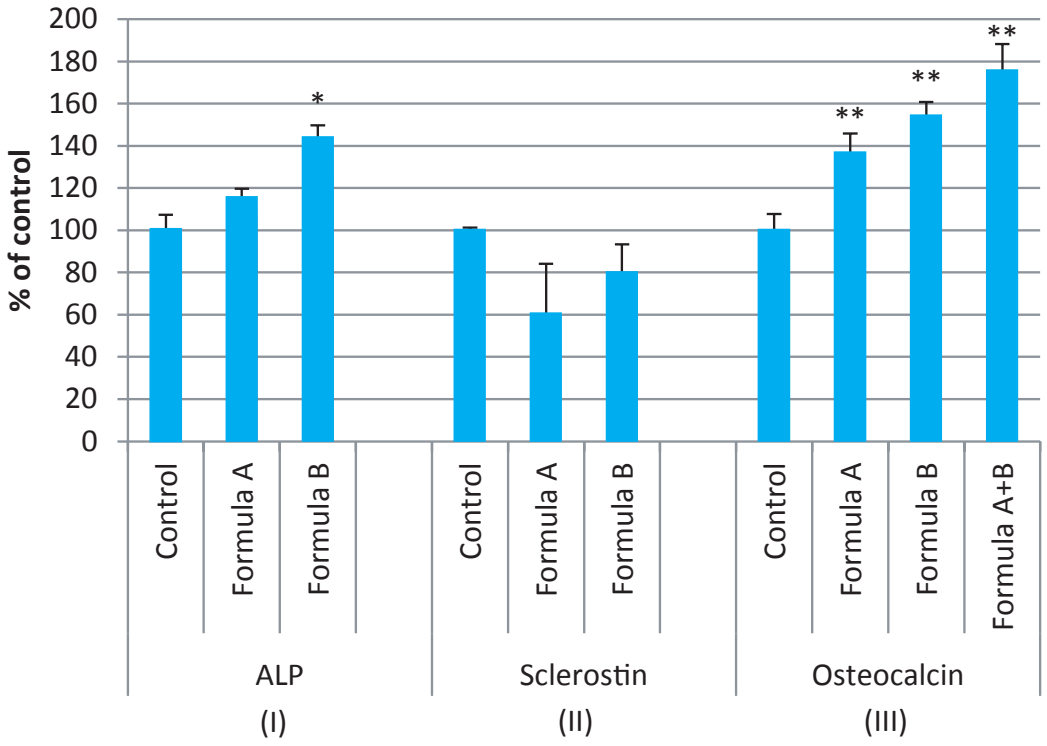

*Figure 2. (I) ALP activity, (II) sclerostin levels, and (III) osteocalcin levels in osteoblasts exposed to Formula A and B at concentrations of 0.125mpdd. Data is presented as the percentage of control. Statistical significance is indicated as *p<0.05;**p<0.01.*

to Formulas A and B have decreased expression of sclerostin by 40% and 20% respectively. Decreased levels of sclerostin indicate improved bone health, as sclerostin inhibits new bone formation and bone remodeling. The results presented on Figure 2-III show that cells applied individually with Formula A and B express significantly higher osteocalcin levels by 37% and 54% respectively. These two formulas combined resulted in even higher increase in osteocalcin—up to 76%—compared to control and individual formulas, which indicates enhanced bone mineralization process in bone-building cells.

Metabolic effects of a multinutrient formula supporting bone health vs basic formula for collagen building: Formula B and Formula C

Optimum mineral supply and collagen formation are important for healthy bones. Therefore, we compared how Formula B (providing important minerals and other factors) and Formula C (containing collagen building blocks) affect different metabolic processes important in bone growth.

The results presented in Figure 3 show that Formulas B and C have positive metabolic effects on bone calcification markers. As such, Formula C—which contains collagen-supporting nutrients—increased ALP activity by 14% compared to control. In the presence of more comprehensive nutrient composition such as in Formula B, the ALP activity in bone cells increased by 43% which was significantly higher compared to control and Formula C (Fig. 3-I). Figure 3-II shows that cells treated with Formula B and C produced 20% and 41% less sclerostin, respectively, than control. A decrease in sclerostin level indicates new bone formation and bone remodeling process. As presented in Figure 3-III, both Formula C and Formula B promoted a significant increase in osteocalcin levels by 49% and 54% respectively compared to control, indicating increased bone mineralization process and bone mineral density.

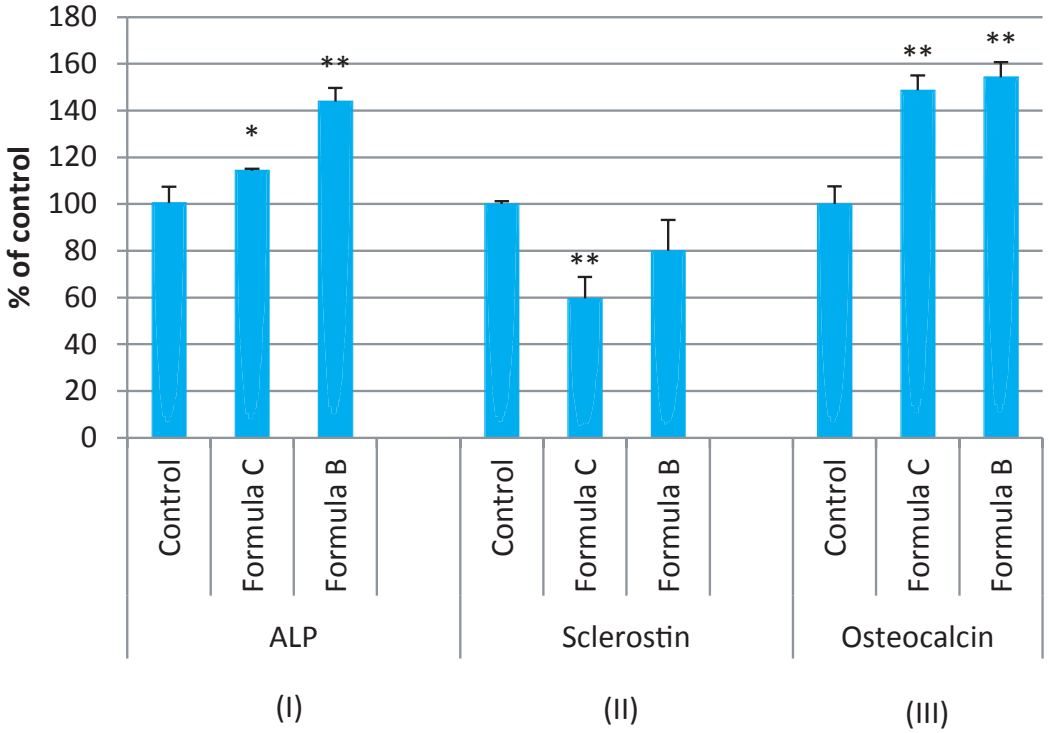

*Figure 3. (I) ALP activity, (II) sclerostin levels, and (III) osteocalcin levels in osteoblasts exposed to Formulas B and C at concentrations of 0.125mpdd. Data is presented as the percentage of control. Statistical significance is represented as *p<0.05;**p<0.01.*

Discussion

The results show that all test micronutrient Formulas—A, B, and C—have positive effects on bone health by targeting critical biological factors that are important at different stages of bone formation. Human bone-building cells treated with these formulas showed an increase in bone growth-promoting factors ALP and osteocalcin, and a decrease in the bone growth inhibitor, sclerostin.

In this study, ALP activity was positively affected by all tested formulas, with the highest efficacy observed with Formula B—a formula enriched with minerals and specific compounds important for bone health—compared to Formulas A and C. Higher ALP activity usually accompanies active bone growth. For example, the level of serum ALP in growing children is 1.5 to 2.5 times higher than in healthy adults.[1] In contrast, a decrease in ALP activity is associated with cessation of bone growth. It is also reported that low ALP activity is related to scurvy, vitamin B12 and vitamin D deficiency, and multi-nutritional deficiency of zinc or magnesium.[2-4]

Osteocalcin, which functions as a bridge between bone matrix and minerals in bone tissues, is a recognized biomarker for bone formation and bone mineralization.[5] It decreases in bone and serum with age, both in men and women, resulting in lower bone mineral density and increased risk of bone fracture.[6] Our results show that all tested formulas applied individually were similarly effective in increasing osteocalcin levels. However, a combination of Formulas A and B was significantly more effective than each formula used individually ($p<0.01$), which would indicate its application both as a support to bone health and as a preventive measure against low bone mineral density.

Interestingly, the formulas with low or no presence of minerals were more effective in decreasing sclerostin levels in osteoblasts than mineral-rich Formula B. For example, we observed that Formula A which contains a comprehensive list of micronutrients, but not a high level of calcium, and Formula C which only contains the essential nutrients for collagen formation, were most effective in decreasing sclerostin levels by 40% and 41% respectively. This was more than the 20% achieved with Formula B, which is rich in minerals and targeted specifically at bone health. As a bone-formation inhibitor, sclerostin negatively regulates osteoblasts differentiation and bone mineralization process, and inhibits bone formation. Higher levels of sclerostin may suggest impaired bone formation, risk of bone fracture, and bone diseases characterized by decreased bone formation.[7] Studies in rodents showed that inhibition of sclerostin improves bone mass, bone strength, and fracture healing.[8] Furthermore, a clinical study on postmenopausal women showed that lowering sclerostin levels improved their bone mass density, reduced serum bone resorption markers, and increased bone formation markers.[9] Serum sclerostin levels are higher in men than in women and increase with age.[10] Because of its important role in bone remodeling, sclerostin has become a potential target for treating osteoporosis and bone fragility.[7]

Our study emphasizes the importance of collagen-supporting micronutrients (Formula C) in various metabolic processes in bone-building cells. The cross-linked collagen helices ensure a strong bone structure for mineralization. In turn, collagen deficiency or its impaired structure may result in bone weakening. Our in vitro results are supported by a clinical study in patients with bone fractures who supplemented with Formula C and citrus bioflavonoids, but did not increase their intake of calcium and vitamin D. The results showed improved well-being and shortening of healing time of fractures by 3 weeks compared to patients taking placebo pills. The results emphasize the importance of nutritional support for optimum collagen formation in bone metabolism when micronutrients such as calcium, minerals and vitamin D are not supplied.[11]

Our bones are constantly undergoing building and remodeling. Different micronutrient combinations in Formulas A, B, and C—both individually and in combination—showed positive effects in regulating

different biological factors involved in specific stages of bone formation. Their synergistic benefits indicate the importance of micronutrient supplementation for optimum bone health.

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