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Effects of Various Multi-Nutrient Supplements on the Production and Extracellular Deposition of Collagen I and IV by Human Aortic Smooth Muscle Cells

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Introduction

Chronic vitamin deficiency is an underlying cause of many modern human chronic diseases, including cardiovascular disease (CVD).[1] A major pathological mechanism leading to the development of CVD is loss of vascular-wall integrity, a structural weakness that triggers the atherosclerotic process.[2] The arterial wall is composed of the constituent cells and surrounding extracellular matrix (ECM).[3]

Smooth muscle cells (SMC), the major cellular component of the arterial wall, contract and relax, altering the luminal diameter, which enables blood vessels to maintain an appropriate blood pressure and to facilitate the redistribution of blood in the body. In addition, SMCs synthesize large amounts of extracellular matrix (ECM), the component responsible for providing the mechanical strength and integrity of the arterial wall, in order to contain the blood and to withstand pulsating pressure waves radiating from the beating heart.[4]

Collagen fibrils form the backbone of the extracellular matrix.[5] The proper ECM assembly in the arterial wall is largely dependent on the quantity and quality of collagen molecules produced and deposited by SMC. It has been known for many years that vitamin C, ascorbic acid, is a key indispensable cofactor of specific enzymes regulating the limiting steps in collagen synthesis.[6]

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Humans lost the ability to produce vitamin C internally, owing to the inactivating mutation of the gulonolactone oxidase gene (GULO), coding for a key enzyme in the pathway of ascorbic acid biosynthesis. Thus, acute vitamin C deficiency in human diet causes scurvy, which is caused by weakening of the arterial wall ECM structure and is manifested by blood leaking through the blood-vessel walls. Although, today, complete vitamin C dietary deprivation is rare, chronic vitamin C insufficiency is quite common in modern society, owing to globalization of agriculture, food processing and storage. Chronic vitamin C deficiency triggers atherosclerotic changes in the vascular wall, which over decades can lead to the development of CVD.[1,7]

Vitamin and mineral supplements are designed to fill the gap in quality of everyday food by providing these and other essential nutrients. However, the composition and quality of vitamin supplements vary widely from manufacturer to manufacturer. Most commercially available multi-nutrient formulas contain randomly selected ingredients in arbitrary doses. This is in contrast to the formulas developed according to synergistic effects of nutrients, an approach pioneered in the work of the Dr Rath Research Institute [www.drrathresearch.org]. Since there are no regulatory requirements demanding scientific proof of the efficacy of nutritional supplements marketed to consumers, many manufacturers promote their products using mere marketing slogans. In best cases, information about the formulas refers to the available published studies, with select individual components contained in the formulation. However, the overall effects of a combination of several nutrients can bring different outcomes from those when each one is used individually.

Therefore, the goal of this study was to determine the efficacy of various popular European and US consumer-market multivitamin formulations, on collagen production and ECM deposition by cultured smooth muscle cells isolated from human aortas.

Materials and Methods

Reagents. All reagents were from Sigma-Aldrich (St. Louis, MO) except when otherwise indicated.

Nutritional Supplements. Seven multivitamin nutritional supplements popular in Germany and other EU countries, and, in some cases, in other international markets, were chosen for the study. They included both lower- and high-cost products. These nutritional supplements were purchased at open market and were at least six months from the claimed expiry date at the time of purchase. All formulas were used within two weeks of purchase and processed as described below. General properties of the supplements in accordance with their corresponding bottle labels are presented in Table 1 (page 3). Commercial names of the products were coded for ethical purposes.

Preparation of Nutritional Supplements. All Nutritional Supplements were treated identically, in accordance with the protocol recommended by the United States Pharmacopeia.[8] Three recommended daily doses of each supplement were taken from freshly opened containers, powdered (tablets were crushed using a ceramic pestle and mortar; capsules were cut open and powder poured out), placed into glass containers with 900 ml of 0.1N hydrochloric acid, and incubated for 1 hour at 37°C in a shaking incubator set with a 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 analyses. Amounts of samples taken for analysis are expressed as number of millionth parts of recommended daily dose of the respective supplement.

Human Aortic Smooth Muscle Cells (AoSMC). Human aortic smooth muscle cells (AoSMC) were purchased from Cambrix (East Rutherford, NJ). Cells were maintained in DMEM medium (ATCC) containing antibiotics and 5% fetal bovine serum (FBS, ATCC). All cell cultures were maintained at 37°C and 5% CO_2 atmosphere. Cell

Table 1 – List of Multivitamin Supplements.

Seven multivitamin nutritional supplements, chosen for the study, were coded as VP, #A, #B, #C, #D, #E and #F. Supplements were analyzed for presence of Ascorbyl Palmitate, Minerals, Amino Acids, Plant Extracts and other components in accordance with container labels.								
	VP	**#A**	**#B**	**#C**	**#D**	**#E**	**#F**	**Average for Formulas #A-F**
Vitamins	14	13	13	13	13	13	13	13
Ascorbyl Palmitate	Yes	No	No	No	No	No	No	No
Minerals	10	11	11	8	10	7	9	9
Amino acids	5	0	0	0	0	0	0	0
Other compounds	2					1	4	2
Plant extracts	2	1	1	1	1	10	1	3

viability was monitored with MTT assay. None of the used experimental conditions resulted in statistically significant cell death (data not shown).

Collagen Production by Human Cultured Cells. For experiments, AoSMC, at 5th to 8th passages, were seeded on collagen type I-covered plastic plates (Becton-Dickinson, collagen I isolated from rat tail tendon) at a density of 25,000/cm^2, and grown to confluence for 2-3 days. Tested compounds were added to cells at indicated concentrations for DMEM, supplemented with 2% FBS. In 72 hours, cell culture media were replaced with fresh supplementations, and cells were incubated for another 48 hours. Cell-produced extracellular matrix was exposed by sequential treatment with 0.5% Triton X100 and 20 mM ammonium sulfate in phosphate-buffered saline (PBS, Life Technologies) for 3 minutes each at room temperature (RT) as described previously.[9, 10] After four washes with PBS, ECM layers were treated with 1% bovine serum albumin (BSA) in PBS for 1 hour at RT and immediately used in experiments.

Collagen Immunoassays. Immunoasssays were done, as described previously,[9, 10] by sequential incubation with primary monoclonal antibodies specific to human collagen type I or IV in 1% BSA/PBS for 2 hours, followed by 1 hour incubation with secondary goat anti-mouse IgG antibodies labeled with horseradish peroxidase (HRP). Retained peroxidase activity was measured after the last washing cycle (three times with 0.1% BSA/PBS), using TMB peroxidase substrate reagent (Rockland). Optical density was read with plate reader (Molecular Devices) at 450 nm. Collagen content was expressed as percentage of control cell samples cultured in plain, unsupplemented 2% FBS/DMEM.

Statistical Analysis. Results in figures are means ± SD from three or more repetitions from the most representative of at least two independent experiments. Differences between samples were estimated with a two-tailed Student's t-test using Microsoft Excel and accepted as significant at p levels less than 0.05.

Results

In the first part of the study we investigated the effects of the multi-nutrient formula (VP) scientifically developed on the basis of the biological synergy of its ingredients on collagen production and ECM deposition by human AoSMC. In independent plates the ECM deposited by AoSMC were analyzed for the content of collagen type I and collagen type IV (Figure 1A and 1B, respectively). Increased concentrations of VP in cell culture medium from 0.3 to 7.2 millionth parts of a daily dose (mpdd) resulted in a dose-dependent increase in the production of both collagen types. Their maximum 8-fold increase was observed at the concentrations of 7.2 mpdd as compared with control cells cultured in a plain cell culture medium.

In the second part of the study we compared the effects of VP to those of six multivitamin supplements originated from other manufacturers in respect to the production of collagen I and IV (Figures 2A and 2B, respectively). All supplements were treated identically and used for supplementation of cell culture medium at the same level of one millionth part of their daily dose. At this level of supplementation only VP and one of the six comparison products expressed statistically significant stimulating effects on collagen type I and type IV production and ECM deposition by cultured human AoSMC. The collagen-production-stimulating effects of VP were statistically superior as compared with other tested multivitamin products.

Discussion

In this study we evaluated how various commercially available multivitamin supplements affect collagen I and IV production by cultured human AoSMC. Although stimulating effects of vitamin C, ascorbic acid, on collagen production by various cell types were reported in numerous studies published previously [reviewed in [11]], it has been also noted that other

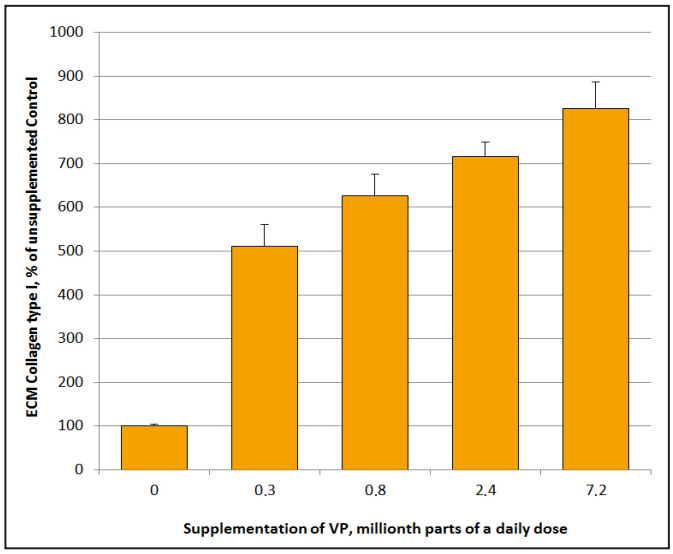

Figure 1A

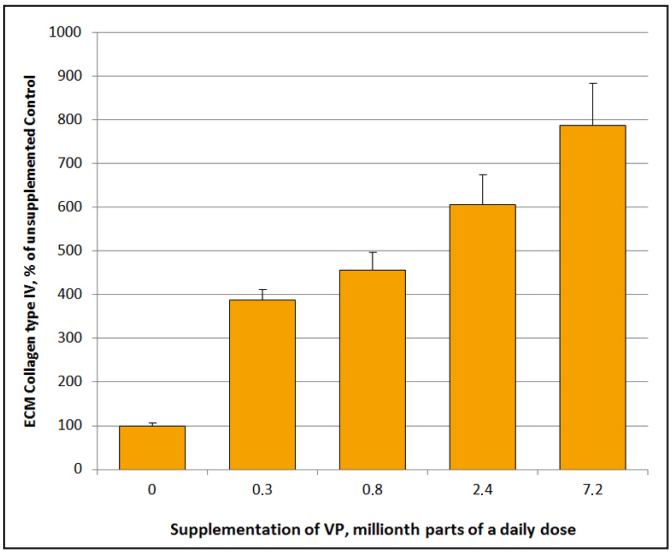

Figure 1B

Figure 1 – Effects of a nutritional supplement (VP) on ECM collagen type I and type IV production and deposition by cultured human aortic smooth muscle cells (AoSMC).

Confluent layers of cultured human AoSMC were incubated in 2% FBS/DMEM containing indicated amounts of multivitamin supplement VP. Cell media were refreshed on day 3. After five day incubation period cell-produced extracellular matrix (ECM) was exposed by differential treatment and assayed for collagen type I (Figure 1A) or collagen type IV (Figure 1B) content by immunoassay. For experimental details refer to Materials and Methods section.

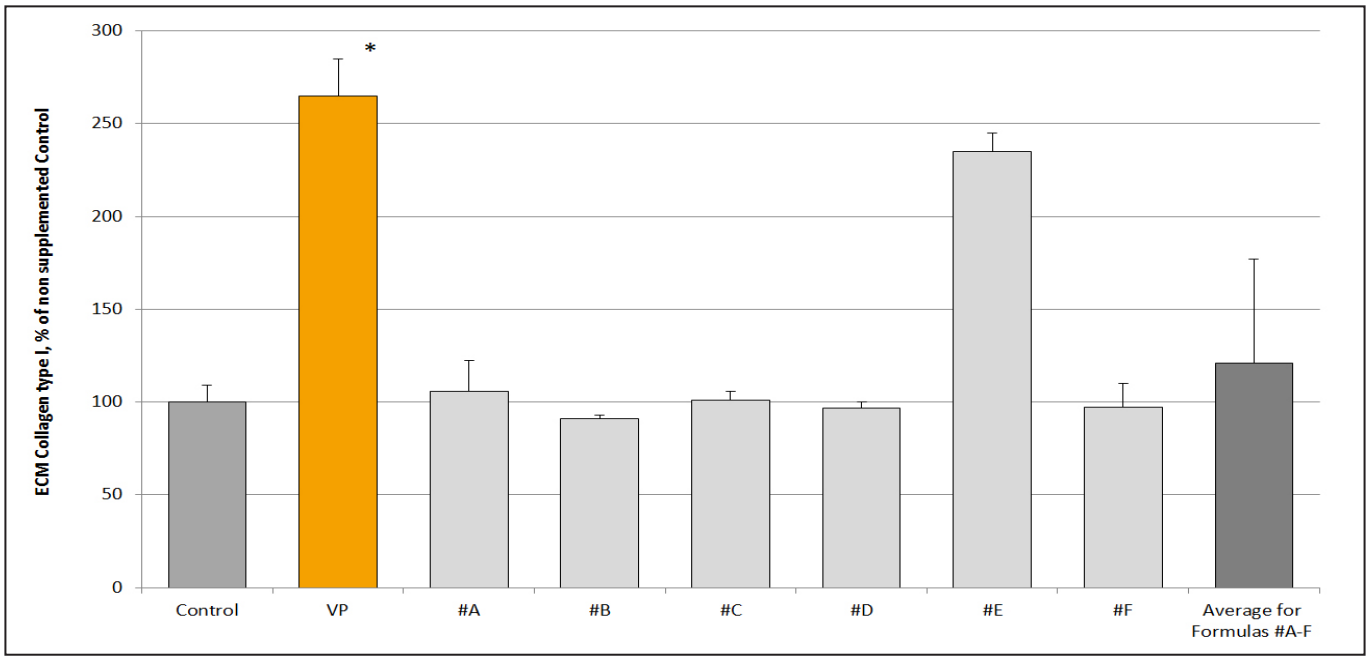

Figure 2A

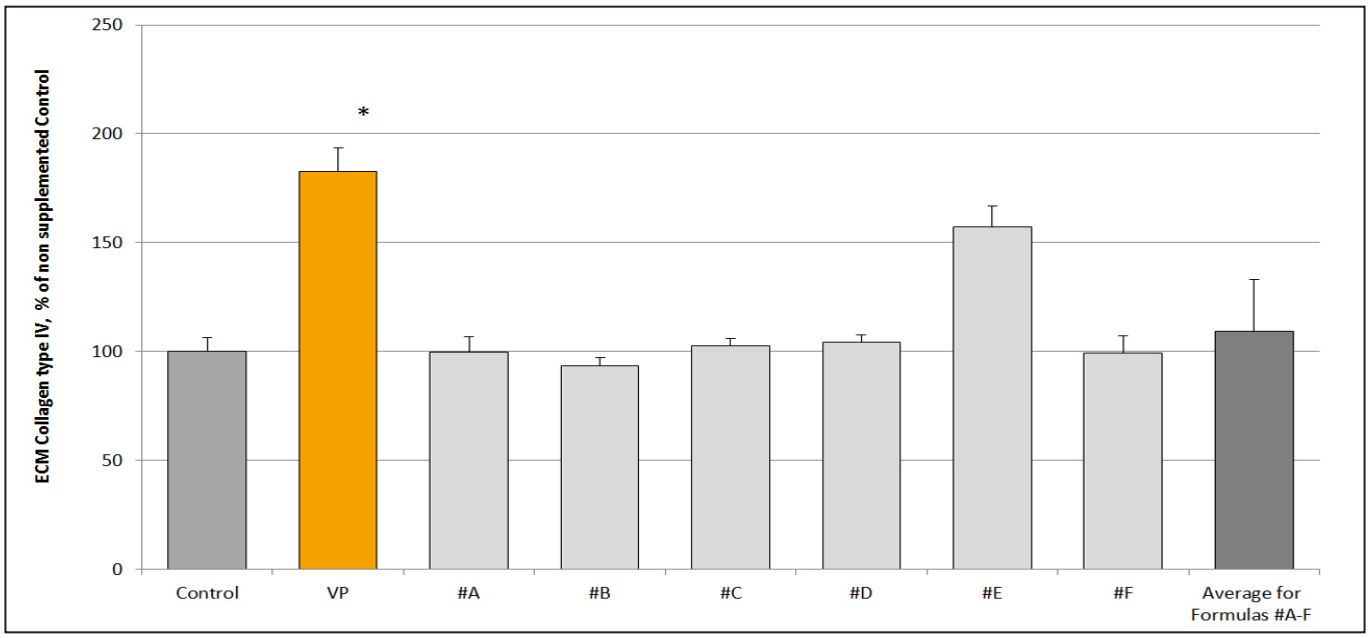

Figure 2B

Figure 2 – The effects of different commercial multivitamin formulations on ECM collagen type I and type IV production and deposition by cultured human aortic smooth muscle cells (AoSMC).

Confluent layers of cultured human aortic smooth muscle cells (AoSMC) were incubated in 2% FBS/DMEM containing one millionth part of a daily dose of each tested commercial supplement. Cell media were refreshed on day 3. After five day incubation period cell-produced extracellular matrix (ECM) was exposed by differential treatment and assayed for collagen type I (Figure 2A) or collagen type IV (Figure 2B) content by immunoassay. For experimental details refer to Materials and Methods section.

** indicates $p<0.05$ when compared to all other products and Control.*

nutrients can modulate this process.[10, 12] Since the production and ECM deposition of collagens are complicated processes involving many steps and stages, different nutrients can influence different parts of these processes. Therefore it is logical to suggest that combinations of nutrients can act either in additive or synergistic manner, when they target the same or different physiological steps, respectively.

We investigated effects of seven commercially available multivitamin supplements with varying ingredient compositions (Table 1). However, except for the presence of five amino acids in the synergy-based VP formula, all tested formulas had a similar composition in respect of the number and types of vitamins, minerals, and all except of formula #E, contained one to two extracts of various plants. The chemical forms of individual nutrients varied, and possibly also the sources of raw materials, as these are not specified on the product labels. The recommended daily dosages of individual components varied between different commercial products. In addition, such factors, as possible differences in the quality (purity) of the materials, presence of inactive fillers and technological conditions of the manufacturing process, also could influence the test results.

Here we demonstrated that multivitamin formulation based on nutrient synergy had superior beneficial stimulating effects on the production by human AoSMC, of two important collagen types, collagen I and IV. Changes in these collagen types' distribution have an impact on mechanical and physiological properties and functions of the artery wall, and play an important role in developing atherosclerosis [reviewed in [13]]. In the normal aorta, the major constituents of the intima, media, and adventitia layer are fibrillary types I and III collagens. Their localization is very similar. Type IV collagen is predominant in the endothelial basement membrane, and in basement membranes of smooth muscle cells of the intima and media layers of healthy arterial wall, important for the integrity of the blood vessels. It is a network-forming collagen that functions as a barrier between different tissue compartments. It has been observed that in fibrous plaques, which are the major form of atherosclerotic lesions of the arterial wall, content of type IV collagens is severely reduced.[14] Therefore optimal content of collagen type IV in the arterial wall tissue is essential for its healthy function and the prevention of the development of atherosclerotic damage.

Although this study does not unequivocally point to the responsible factor for the higher efficacy of VP in supporting collagen synthesis by smooth muscle cells, it appears that the presence of amino acids, some of which are important components of collagen molecules, as well as the dosages and chemical forms of ingredients, may play an important role in the enhanced efficacy of the synergy-based VP formulation. For instance, we looked into the vitamin C content, as it is a critical nutrient in collagen synthesis. Based on the daily recommended intake of the test product, it would be equal to 80 mg of vitamin C in products #A, #C, #D, 100-120 mg in #B and #F, and 310 in formula #E. The vitamin C content in the formula VP was 600 mg, and, unlike in the other products, about 1/3rd of it was as ascorbyl palmitate, a fat-soluble form of vitamin C.

The results would imply that VP formulation may provide superior health benefits compared with other tested commercially available formulas in supporting strength and overall health of blood vessels and cardiovascular system function.

These results as well as those cited in our earlier publications on this topic[15, 16] clearly stress the need for scientific testing in product formulations. This includes demanding from the manufacturers of nutritional supplement products, scientific proofs and confirmation of efficacy of marketed formulas. This will guarantee that vitamin consumers are assured of the safety and efficacy of natural products they use, and will increase the scientific legitimacy and prestige of the natural health industry.

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