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EFFECTS OF LIFEPAK® SUPPLEMENTATION ON ANTIOXIDANT STATUS AND LDL-OXIDATION IN HEALTHY NON-SMOKERS

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ABSTRACT

Background: Although there is substantial evidence for antioxidant effects of individual nutrients, such as vitamins C and E, little research has been done to show the antioxidant efficacy of nutritionally complete micronutrient supplements. **Objective:** This study examined whether a comprehensive multivitamin/mineral supplement (MVMS; LifePak®) improves antioxidant status and resistance to LDL oxidation in healthy non-smokers. **Methods:** In this double-blind, randomized crossover study, 50 subjects received MVMS and placebo (PL) for 6 weeks with a 6-week washout period. MVMS provided vitamin C, vitamin E, carotenoids and flavonoids among other nutrients. **Results:** MVMS significantly increased serum concentrations of ascorbic acid (from 68.1 to 94.3 μmol/L) β-carotene (from 335 to 716 nmol/L), α-carotene (from 77 to 592 nmol/L), and vitamin E (α-tocopherol, from 20.0 to 36.9 μmol/L), with no changes in PL treatment. MVMS significantly decreased LDL oxidizability, as the lag time was prolonged (by 17 %), and oxidation rate was reduced without changes with PL treatment. **Conclusions:** MVMS significantly increased serum antioxidants and decreased LDL oxidizability. Results suggest that MVMS supplementation may have cardiovascular and antioxidant benefits in healthy non-smokers.

Keywords: Multivitamin, Antioxidants, Atherosclerosis, Cardiovascular Disease, Carotenoids, α-Carotene, β-Carotene, α-Tocopherol, Vitamin C

INTRODUCTION

Aerobic metabolism and environmental factors can generate a variety of reactive oxygen species (ROS), which can damage DNA, as well as structural and functional proteins, carbohydrates, and lipids (1,2). ROS have been implicated in the pathogenesis of many chronic diseases, including atherosclerosis (3-6). Some of the health-promoting effects of antioxidant nutrients, such as the vitamins C and E, carotenoids, and flavonoids, are thought to be based on their ability to quench ROS (6-8).

Numerous clinical studies have demonstrated the health benefits of individual antioxidant nutrients, and more recently, a few human studies have addressed the effects of limited combinations of antioxidant nutrients (9-11). Studying the effects of more complete antioxidant nutrient combinations is a worthwhile endeavor, because most of these nutrients closely interact with each other and with the body's intrinsic antioxidant systems to provide protection from free radical damage (12-15). Consequently, it is safe to assume that the best overall protection from free radical damage and pathologic conditions may be achieved by consuming diets and supplements rich in a variety of antioxidant nutrients (7).

However, little human research has been done on dietary supplements combining more than three antioxidant nutrients. An extensive on-line literature search in the Medline database (1966 - present) yielded no human prospective studies testing combinations of vitamins C and E, selenium, carotenoids and flavonoids. Girodon et al. tested a combination of vitamins C and E, β-carotene, zinc and selenium in amounts close to the RDA in French hospitalized elderly, and showed improved antioxidant status (9). Likewise, Preziosi et al. supplemented 201 middle-aged healthy subjects in a multi-center study with a similar antioxidant combination, and showed improvements in biochemical indicators of antioxidant status (10). Jialal and Grundy supplemented men with a high-dose combination of α-tocopherol, β-carotene and vitamin C versus α-tocopherol alone for three months and observed similar reductions of LDL oxidizability in both groups, concluding that the addition of β-carotene and vitamin C did not enhance α-tocopherol's efficacy (16).

The present study was designed to evaluate the antioxidant effects of a commercially available, comprehensive multivitamin/mineral supplement with above-RDA amounts of vitamins C and E (i.e., 500 mg and 300 IU, respectively) and other antioxidant nutrients, such as carotenoids, flavonoids, the trace element cofactors for antioxidant enzymes (Cu, Zn, Mn, and Se) and all other essential micronutrients. The present study determined the effects of this micronutrient supplement on antioxidant status and resistance against LDL oxidation in healthy adult non-smokers.

METHODS AND MATERIALS

Subjects. Included in this study were 50 healthy male and female subjects between 18 and 65 years of age recruited by GFI Pharmaceutical Services, Inc. in the Evansville, Indiana, area

(USA). The number of subjects was estimated using statistical power calculations based on data from existing vitamin E supplementation studies. All subjects were healthy non-smokers consuming typical U.S. diets with less than five daily servings of fruits and vegetables. Good health was determined by physical exam, medical history and clinical laboratory evaluation. Except for the test product, subjects did not take any dietary supplements or drugs with potential antioxidant or oxidant effects three months prior to or during the study. Subjects also did not consume more than one serving of alcoholic beverages daily on average and had no evidence or history of substance abuse. They were instructed and willing to maintain their usual dietary and exercise habits throughout the duration of the study. Female subjects were not pregnant or nursing and had to use adequate birth control precautions, and postmenopausal women receiving hormone replacement therapy were instructed to remain at the same dosage throughout the study.

Treatments. This study was carried out as a double-blind, randomized cross-over design with two six-week treatment periods and a six-week washout period in between. The study protocol, informed consent forms and final report were reviewed and approved by an Institutional Review Board (Ohio Valley IRB). The 50 subjects were randomly assigned to receive either a commercially available comprehensive multi-vitamin/mineral supplement (MVMS; LifePak®, Pharmanex, Provo, Utah, U.S.A.) or an identically appearing placebo (PL). Both treatment and placebo supplements were manufactured under Good Manufacturing Practices. The composition of MVMS is shown in **Table 1**. After the washout period, treatments were reversed for each subject. The study was conducted during the months of December-April. Subjects were instructed to take one packet (containing 3 capsules) of MVMS or PL twice daily, i.e., with their morning and evening meals. Safety was assessed by physical examination, clinical laboratory evaluation (blood chemistry, hematology and urinalysis) and by monitoring of adverse reactions. The conduct of the study at the contract research site was monitored by an independent clinical study monitor.

Measurements. At the beginning and end of each treatment period, serum and plasma samples were collected after an overnight fasting period, and assayed to determine antioxidant status and LDL oxidizability. A separate serum sample for ascorbate analysis was collected and preserved using a 1:1 dilution with freshly prepared meta-phosphoric acid, 10%. All serum and plasma samples were stored at -70 °C. At the end of the clinical treatments, sera and plasma samples were shipped overnight on dry ice to the analytical laboratories. Serum ascorbate was determined using a Cobas Fara II spectrofluorometric analyzer (Roche Diagnostic System, Inc., Branchburg, NJ) by a colorimetric, ferrozine-based reaction after TCA protein precipitation (modified McGown et al. 1982 method). Serum uric acid was determined using the Cobas Fara II analyzer by an enzymatic colorimetric method based on uricase, peroxidase and 4-aminoantipyrine (Sigma Chemical Co., St. Louis, MO). Serum selenium and iron were determined using atomic absorption spectrophotometry. Serum lipid-soluble antioxidants (β- and α-carotene, α-tocopherol, retinol and retinyl palmitate) were analyzed by a modified C_{18} reversed phase HPLC method of Epler et al. (17) using isocratic mobile phase, diode array detection for retinol and carotenoids and fluorescence detection for the tocopherols. Internal standards (NIST) were used for quantitation, after ethanolic protein denaturation and hexane extraction. LDL oxidizability was measured at the Center for Human Nutrition, University of Texas Southwestern Medical Center, Dallas Texas, as described by Marangon et al. (19). Briefly, LDL were isolated by ultra-centrifugation, passed through a Biorad® column and protein was estimated by the Lowry method. Thereafter, copper-

catalyzed LDL oxidation was carried out with 100 μg/mL LDL protein and 2.5 μM copper at 37 °C

TABLE 1
Nutrient Composition of MVMS (LifePak®).

Six capsules (= 1 daily supply in 2 packets) provide:	Amount		% DV[1]
Vitamin A (retinyl palmitate), 5,000 IU	1.5	mg	100
β-Carotene (from palm fruit extract and *Dunaliella salina*), 10,000 IU	6	mg	200
Vitamin C (calcium ascorbate)	500	mg	833
Vitamin D_3 (cholecalciferol), 400 IU	10	μg	100
Vitamin E (*d*-α-tocopherol (92% succinate, 8% non-esterified), 300 IU	205	mg	1,000
d-β/γ/δ-tocopherols and tocotrienols (non-esterified)	4.7	mg	n/a
Thiamin (mononitrate)	3	mg	200
Riboflavin	3.4	mg	200
Niacin (niacin, niacinamide)	40	mg	200
Vitamin B_6 (pyridoxine hydrochloride)	10	mg	500
Folate (folic acid)	600	μg	150
Vitamin B_{12} (cyanocobalamin)	30	μg	500
Biotin	300	μg	100
Pantothenic Acid (D-calcium pantothenate)	30	mg	200
Vitamin K_1 (phylloquinone)	40	μg	50
Calcium (carbonate, citrate, propionate, glycine chelate)	500	mg	50
Magnesium (aspartate, oxide, glycine chelate)	250	mg	63
Iron (glycine chelate)	3	mg	17
Iodine (potassium iodide)	75	μg	50
Zinc (glycine chelate)	15	mg	100
Copper (glycine chelate)	2	mg	100
Manganese (glycine chelate)	3.6	mg	180
Selenium (L-selenomethionine, sodium selenite)	100	μg	143
Chromium (glycine niacin chelate)	200	μg	167
Molybdenum (glycine chelate)	75	μg	100
Vanadium (vanadyl sulfate)	20	μg	n/a
Silicon (sodium metasilicate)	3	mg	n/a
Boron (citrate)	3	mg	n/a
α-Carotene (from palm fruit extract)	2	mg	n/a
Lutein (from marigold flower extract)	2	mg	n/a
Lycopene (from tomato extract)	1	mg	n/a
α-Lipoic Acid	10	mg	n/a
Broccoli and Cabbage Extracts (20:1, with glucosinolates)	100	mg	n/a
Quercetin	50	mg	n/a
Citrus Bioflavonoids (hesperidin, naringenin)	25	mg	n/a
Grape Seed Extract (min. 92% polyphenols)	10	mg	n/a
Curcumin (from turmeric extract, 95%)	50	mg	n/a
Soy Isoflavones (from soy isoflavone extract, 40%)	10	mg	n/a

[1]U.S. Food and Drug Administration, Daily Values for nutrition labeling.

for a five-hour period. Conjugated diene formation was monitored in ten-minute intervals at 234 nm. From these data, the lag phase and the oxidation rate were calculated. Clinical laboratory

evaluations were performed by a CLIA approved clinical laboratory at GFI Pharmaceutical Services, Inc.. Total fruit and vegetable consumption was assessed by a dietician using a 7-day food diary recorded by the subjects during the 3rd week of each treatment period. Intakes of specific fruits and vegetables were not assessed.

Statistical analyses. Subject demographics and baseline characteristics were tabulated and compared between the treatment groups using two-sample t-tests and Fisher's Exact tests. Changes in antioxidant status and LDL-oxidation variables were compared between the product groups using ANOVA for a two-period crossover design. The statistical model contained the main effect of sequence, subject within sequence, period and product. Sequence effects were tested using the subject within sequence as the error term. The period and product effects were tested using the residual error. The analysis was conducted on the raw day-0 to day-42 change and the percent change from day 0 to day 42. Summary statistics of changes in vital signs from screening to endpoint were tabulated. Changes in quantitative clinical laboratory variables were computed and the product groups were compared using the previously described two-period crossover ANOVA. Results are expressed as means ± SD. All data listings and statistical summations were performed using the Statistical Analysis System (SAS®), version 6.12.

RESULTS

Four out of the 50 subjects enrolled in this study dropped out. Reasons for dropout were adverse effects (hives, itching) due to PL (1 subject), pregnancy (1 subject), address change (1 subject) and non-compliance (1 subject). A total of 46 subjects were available for the baseline to endpoint analyses. The mean age of the 46 subjects that completed the study was 40.0 years (range: 20 - 65), and 72 % of the subjects were female. The mean body weight (± SD) for all subjects was 77.4 ± 19.2 kg. Subject demographics, as well as mean body weight and body mass index (BMI) for each treatment sequence are listed in **Table 2**.

TABLE 2
Subject Demographics and Vital Signs at Screening Visit

Characteristic		Sequence 1 (n = 24)	Sequence 2 (n = 22)
Sex	Male	9 (38 %)	4 (18 %)
	Female	15 (62 %)	18 (82 %)
Age[1] (y)		39.1 ± 12.0	40.9 ± 11.4
Range (y)		25 - 65	20 - 62
Body Weight[1] (kg)		76.3 ± 19.1	78.7 ± 19.8
Body Mass Index[1] (kg/m^2)		26.6 ± 4.8	27.3 ± 6.5

[1]means ± SD.

Body weight and BMI were not significantly different between treatment sequences. Systolic and diastolic blood pressure, other vital signs, and all blood chemistry, hematology and urinalysis measurements were normal at study initiation and remained normal throughout the study. Mean compliance in terms of product accountability for MVMS and PL were 94.7 ± 4.6 % and 92.8 ± 5.6 %, respectively. This difference was statistically significant ($p = 0.012$). No severe adverse events were reported during the study. The only event judged to be possibly related to product was hives and itching after five days of receiving PL product. The average fruit and vegetable consumption obtained from 7-day food diaries recorded during the third week of each treatment period remained unchanged between treatment periods ($p = 0.82$) and between treatments (MVMS: 2.7 ± 0.9; PL: 2.7 ± 1.1; $p = 0.80$). Results confirmed that the 6-week washout and treatment periods in this cross-over study appeared to be sufficiently long to ensure complete return to baseline concentrations of all variables measured in this study, except α-carotene which showed a small but significant carry-over effect.

TABLE 3

Changes from Baseline to Endpoint in Levels of Serum Antioxidants and Iron
(means ± SD; n = 46)

Variable	MVMS		PL	
	Baseline	Endpoint	Baseline	Endpoint
Vitamin E				
α-Tocopherol (μmol/L)	20.0 ± 8.5	36.9 ± 13.0[c]	19.9 ± 6.8	20.6 ± 9.0
(μmol/mmol serum lipid[1])	3.3 ± 0.85	6.2 ± 1.48[c]	3.4 ± 0.72	3.5 ± 0.86
Vitamin C (ascorbate, μmol/L)	68.1 ± 24.8	94.3 ± 26.4[c]	67.4 ± 21.4	70.6 ± 24.2
Carotenoids				
β-Carotene (nmol/L)	335 ± 198	716 ± 429[c]	331 ± 178	299 ± 131
(nmol/mmol serum lipid[1])	62.7 ± 35.0	140.2 ± 100.1[c]	63.1 ± 30.1	58.3 ± 29.8
α-Carotene (nmol/L)	76.6 ± 81.7	592 ± 365[c]	121 ± 104[d]	84.9 ± 89.1
(nmol/mmol serum lipid[1])	13.9 ± 14.2	113.0 ± 72.6[c]	23.2 ± 17.6[d]	16.9 ± 17.5
Vitamin A				
Retinol (μmol/L)	2.09 ± 0.61	2.18 ± 0.48	1.99 ± 0.54	2.00 ± 0.55
Retinyl palmitate (nmol/L)	22.8 ± 31.5	91.9 ± 166.8[a]	24.0 ± 31.2	30.6 ± 35.8
Selenium (μmol/L)	2.10 ± 0.41	2.47 ± 0.75[b]	2.15 ± 0.39	2.26 ± 0.63
Iron (total, μmol/L)	22.8 ± 6.6	21.5 ± 8.3	23.1 ± 8.9	22.2 ± 8.4
Uric Acid (μmol/L)	287 ± 85	285 ± 912	287 ± 83	287 ± 83

[1]calculated as: serum concentration / (mmol/L total cholesterol + mmol/L total triacylglycerols)
[a]$p \leq 0.05$, significant difference in change from baseline to endpoint in supplement compared to placebo.
[b]$p \leq 0.01$, significant difference in change from baseline to endpoint in supplement compared to placebo.
[c]$p \leq 0.001$, significant difference in change from baseline to endpoint in supplement compared to placebo.
[d]$p \leq 0.05$, significant difference between supplement and placebo at baseline.

Changes in serum antioxidant concentrations are shown in **Table 3**. The six-week treatment with MVMS caused a significant 84.5 % increase in serum α-tocopherol ($p \leq 0.001$) concentrations. Serum-lipid-normalized α-tocopherol concentrations showed similar changes as

serum α-tocopherol. Serum ascorbate concentrations were significantly increased by 38 % ($p \leq 0.001$) with MVMS without changes in the PL period. MVMS elevated serum retinyl palmitate four-fold ($p \leq 0.001$), whereas retinol concentrations remained unchanged ($p = 0.321$). Among the serum carotenoids, β-carotene and α-carotene showed significant 2.1 and 7.7-fold increases ($p \leq 0.001$). Serum total selenium concentrations increased significantly by 17 % ($p = 0.007$) with MVMS. No changes in any of these serum measurements were observed during the PL period.

Results of the copper-catalyzed *ex vivo* LDL oxidation assays are shown in **Table 4**. The lag time of conjugated diene formation was significantly prolonged by 17 % ($p \leq 0.001$) with MVMS, whereas no changes were observed with PL. Likewise, the oxidation rate was significantly decreased by MVMS ($p \leq 0.001$) without effects during the PL period. At the same time, serum total cholesterol, LDL cholesterol, HDL cholesterol and triacylglycerol concentrations remained normal and unchanged with both treatments during the study (**Table 4**).

TABLE 4
Baseline-to-Endpoint Changes in LDL Oxidizability and Blood Lipids
(means ± SD; n = 46)

	MVMS		PL	
Variable	Baseline	Endpoint	Baseline	Endpoint
LDL oxidizability				
Lag time (min)	43.4 ± 6.3	50.9 ± 8.6[a]	42.1 ± 7.3	42.0 ± 5.9
Ox. rate (μmol/min×g protein)	12.5 ± 1.8	11.4 ± 1.7[a]	12.7 ± 1.6	12.9 ± 1.6
Blood Lipids (mmol/L)				
Total cholesterol	4.74 ± 1.00	4.55 ± 0.85	4.67 ± 0.93	4.57 ± 0.89
LDL cholesterol	2.83 ± 0.79	2.60 ± 0.79	2.80 ± 0.74	2.67 ± 0.67
HDL cholesterol	1.50 ± 0.42	1.51 ± 0.46	1.48 ± 0.41	1.49 ± 0.43
Triacylglycerols	0.88 ± 0.42	0.97 ± 0.47	0.86 ± 0.46	0.91 ± 0.44

[a] $p \leq 0.001$, significant difference in change from baseline to endpoint in supplement compared to placebo.

There were significant correlations of baseline-to-endpoint changes in serum-lipid-normalized concentrations of α-tocopherol ($R = 0.56$, $p = 0.0002$), α-carotene ($R = 0.50$, $p = 0.0009$) and β-carotene ($R = 0.36$, $p = 0.0227$), as well as serum ascorbate ($R = 0.33$, $p = 0.0270$) with the changes in lag time of LDL oxidizability. No significant correlations were observed of serum antioxidants and LDL oxidation rate.

DISCUSSION

To the best of our knowledge, this is the first published study that tested antioxidant effects of a nutritionally complete micronutrient supplement in healthy adults. MVMS provided RDA-based amounts of most vitamins and minerals, and higher amounts of those nutrients that have demonstrated clinical benefits at above-RDA dietary intakes, such as vitamins C and E. In

addition, MVMS also included several non-essential antioxidant micronutrients in the carotenoid and flavonoid categories. The complete composition of MVMS is shown in **Table 1**.

Serum Vitamin E. Vitamin E supplementation has been shown to reduce the progression of atherosclerosis (20-22) and to inhibit LDL oxidizability (23). Whereas U.S. vitamin E intakes appear to be slightly below the RDA of 8-10 mg/d (12-15 IU/d) (24), current recommendations for optimum vitamin E daily intakes range from 100 to 250 IU/d (25,26). Daily amounts of 800 IU are considered safe for long-term supplementation (27,28). MVMS provided 201 mg (300 IU) of vitamin E from RRR-α-tocopherol and other natural tocopherols. Serum α-tocopherol baseline concentrations and the observed 84.5 % increase with MVMS in the present study (**Table 3**) are in agreement with the changes shown in other human studies using similar doses and duration (23-30).

Serum Vitamin C. Vitamin C supplementation above the current RDA of 60 mg shows promising effects in preventing cataract (31) and cardiovascular disease (32,33), and as adjunct therapy in diabetics (34). Recent studies suggest that the optimum dietary intake of vitamin C may be between 200 and 500 mg/d, and that intakes over 500 mg/d may have little or no additional health benefits (35,36). Serum vitamin C concentrations (**Table 3**) increased significantly with MVMS which provided 500 mg of ascorbate, even though serum baseline and PL ascorbate were at concentrations indicating above-RDA dietary intakes (35,37). Because baseline ascorbate concentrations already approached saturation concentrations (35,37), the observed increase with MVMS (by 26.2 μmol/L to 94.3 ± 26.4 μmol/L) is remarkable. With the observed mean consumption of fruits and vegetables of only 2.7 servings/day, and no data on individual fruit and vegetable intake, we can only speculate that the high vitamin C intakes may have come from popular fortified foods and beverages. In addition, a major portion of the fruit and vegetable intake by the study population could have come from citrus fruit, which is typically abundant during the time of year this study was conducted (December-April).

Serum Carotenoids. Many carotenoids promote immune function and may prevent cancer (38-40). Carotenoid intakes in the U.S. population are considered low and reflect low fruit and vegetable consumption (41). The amounts of carotenoids present in MVMS resemble dietary intakes from diets with the recommended five servings of fruits and vegetables (**Table 1**). The significant 114 % increase in serum β-carotene (**Table 3**) observed in the present study with 6 mg/d β-carotene confirms data reported in the previously published literature (9,11). The 7.7-fold increase in serum α-carotene concentration observed with MVMS supplementation (**Table 3)** providing 2 mg/d of α-carotene confirm existing data showing serum α-carotene responds well to dietary supplementation, even in the presence of large amounts of other carotenoids (11,42). Overall, the present study did not show strong evidence of potentially adverse interactions among the supplemented carotenoids, and thus confirmed similar conclusions by Mayne et al. (42).

Serum Retinol and Selenium. The present study confirms that in healthy, well-nourished subjects serum retinol concentrations remain largely unaffected by vitamin A supplementation due to tight homeostatic control. However, serum retinyl palmitate was increased significantly due to the vitamin A (1.5 mg) provided by MVMS as retinyl palmitate. MVMS provided 100 μg/d of

selenium, 50 % from sodium selenite and 50 % from L-selenomethionine. Compared with other selenium supplementation studies (9,43-45), baseline serum selenium concentrations in this study population were somewhat high. Nevertheless, MVMS increased serum selenium concentration by 18 % (**Table 3**).

LDL Oxidizability. There is now substantial evidence available to support the hypothesis that oxidatively modified LDL play a major role in atherogenesis (6,23,46), and that antioxidant nutrients that inhibit LDL oxidation, such as vitamin E, also slow the progression of atherosclerosis (20-22). In an 8-week dose-response study of vitamin E supplementation and LDL oxidation in healthy men, Jialal et al. (23) found significant increases in the lag time of conjugated diene formation with 400 to 1200 IU/d vitamin E, but not with 200 IU/d. Kruger et al. (47) showed in a single-blind placebo-controlled study of hypercholesterolemic men that a combination of 87 IU/d vitamin E, 300 mg/d ascorbic acid, 8.85 mg/d β-carotene, and 90 μg/d selenium increased the lag time of LDL oxidation by 10.6 % after 6 months of supplementation. The significant 17 % increase in lag time and the 9 % decrease in oxidation rate observed in the present 6-week study (Table 4) confirm that MVMS's antioxidant combination effectively improved the resistance of LDL against oxidative damage in healthy adults. Consequently, MVMS may have important cardiovascular health benefits.

LDL oxidizability was significantly correlated with lipid-normalized serum concentrations of α-tocopherol, α-carotene and β-carotene, as well as serum ascorbate. Judging from the degree of correlation, it appears that α-carotene may be a more important antioxidant than β-carotene or ascorbate in inhibiting LDL oxidation. A role for α-carotene in reducing LDL and plasma oxidizability was also suggested by others (48) and warrants further studies.

Conclusions. The present study showed that MVMS significantly increased antioxidant status as measured by serum antioxidant levels, and improved resistance against LDL oxidation. Therefore, MVMS may have cardiovascular benefits in healthy adults consuming typical U.S. diets. The findings also confirmed two widely held assumptions: first, that a more complex antioxidant nutrient combination from 12 different sources is efficacious, and second, that the tested combination is efficacious in the presence of a full spectrum of non-antioxidant nutrients of a nutritionally complete micronutrient supplement.

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