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# Effects of multinutrient supplementation on antioxidant defense systems in healthy human beings

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**Abstract**

Oxidative damage involved in the pathogenesis of many diseases, such as cardiovascular disease, cancer and diabetics. The antioxidant defense system plays an important role in protecting body from oxidative damage. Numerous studies have been shown that a single vitamin or mineral supplementation has the beneficial effect on the antioxidant defense system. However, the overall combined effect of multinutrient supplementation on antioxidant defense system remains to be clarified. In the present double blind, placebo-controlled study, the antioxidative defense system was measured in 34 healthy subjects before and after multinutrient supplementation. Plasma vitamin C, E and $\beta$-carotene, erythrocyte vitamin E, as well as whole blood selenium all showed increase at 5 weeks of supplementation. The activities of catalase (CAT) and glutathione peroxidase (GPX), but not superoxide dismutase (SOD), as well as GSH level were significantly increased at 16 weeks of supplementation. Moreover, the resistance of erythrocytes to 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH)-induced oxidation was elevated at 5 weeks after supplementation. These results clearly demonstrated that short-term supplementation (16 weeks) with multinutrient could markedly improve antioxidative vitamin status and enzymatic activities. These improvements also led to the reduction of RBC susceptibility to free radial peroxidation. 

*Keywords:* Antioxidant; Antioxidative enzymes; Vitamin; Mineral; Supplement; Nutrition

## 1. Introduction

Free radical induced lipid oxidation in human bodies has been related to the pathological process of many diseases, such as cardiovascular disease [1,2], some cancerous disorders [3] and diabetics [4]. The research on biological antioxidants, therefore, has attracted great attention from both the scientific community and the general public.

As an effort to improve one's health status and maintain the optimal body function, multinutrient supplementation has become a more and more popular practice nowadays in Europe and North America. Many of the ingredients in those commercial supplement packs have been individually proven to have direct or indirect effect on cellular antioxidative systems. Besides vitamin C, E, and $\beta$-carotene, of which the effects as biological antioxidants have been well confirmed [5,6]. Recent studies also indicate that some B vitamins may also play important roles in the cellular antioxidation defense systems [7,8]. The effects of trace minerals, such as Fe, Cu, Mn, and Zn, on antioxidative enzymes, catalase (CAT), superoxide dismutase (SOD), have also been well established [9]. Recently, selenium (Se) has received large amount of attention because of its role in the formation of cellular glutathione peroxidase (GPX) [10].

Some vitamins are known to show synergistic effect if they exist together as antioxidants [11,12]. This kind of synergistic effect may be expressed as the beneficial influence on blood lipids as indicated in the study of Morcos [13], who demonstrated that oral intake of a full complement of multivitamin and multimineral can favorably modify lipid profile and reduce the risk of cardiovascular diseases. The overall combined effect of a dietary supplement upon the anti- and pro-oxidation balance in human body, therefore, becomes more meaningful to inquire than the individual effect of different components.

Although many efforts have been made to study the effect of vitamin and mineral supplementation on human antioxidation systems, most of them were focused upon a single substrate or a simple combination of a few substrates. The subjects studied were also limited to a particular type of

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population, such as smokers [14], elders [15], and AIDS patients [16]. The aim of the present study, therefore, is to examine the overall effect of a comprehensive supplementation of multinutrient on the antioxidant defense systems in healthy persons.

## 2. Materials and methods

### 2.1. Subjects

Thirty four healthy persons of normal lifestyle, 21 males and 13 females, with a mean age of 24.6 (range 19–46) years, were recruited from a college community. Each subject gave written informed consent to participate in the study. Those subjects were instructed to maintain their regular lifestyle, report every noticeable change as well as continue their usual daily diets and activity levels during the study. Adherence to the same diet was evaluated haemodinamically by measurement of systolic and diastolic blood pressure, and metabolically and nutritionally by measurement of serum total protein and albumin. No significant changes were observed during the study period. Moreover, none of the subjects were taking other nutrient supplements during study period. Subjects were divided into two groups, 17 each, with age and gender carefully matched. Supplement was administrated in a double-blind manner. At the indicated sampling points, which were the 2nd week before supplementation (−2 week), the starting day (0 week) and the 5th and 16th week after starting on supplement (5 and 16 week), fasting peripheral blood was collected into EDTA blood sampling tubes and, after an overnight storage at 4°C, were subjected to chemical and biochemical analyses as described below. The RBC suspensions were prepared from phosphate-buffered saline (PBS)-washed cells after removal of plasma and three serial washing with PBS at 4°C. Cells were then resuspended into the original volume of blood sample with PBS. Erythrocyte hemolysates were obtained by adding 4 volumes of distilled water into 1 volume of RBC suspension. The hemolysates were frozen in aliquots at −80°C for later analysis of glutathione and enzymatic activity.

### 2.2. Materials

Multinutrient daily packs (brand name *DailyGuard*) were obtained from Viva Life Science, Costa Mesa, CA, USA. This product contained a full complement of multivitamins, multiminerals and herbs (Table 1) and was formulated using natural ingredients in a starch base. Tablets were pressed using low compression pressures of approximately 60,000 lb to facilitate gastric disintegration. The placebo group received tablets of similar appearance containing inert cellulose filler in a starch base.

Table 1
Contents of daily supplements

| | |
|---|---|
| Vitamin A | 14,000 U (11,350 U from β-carotene) |
| Vitamin C (ascorbic acid) | 535.0 mg |
| Vitamin D | 275 U |
| Vitamin E (vitamin E acetate) | 176 U |
| Folic acid | 212 mcg |
| Vitamin B1 (thiamine) | 1.7 mg |
| Vitamin B2 (riboflavin) | 3.3 mg |
| Vitamin B3 (niacinamide) | 15.8 mg |
| Vitamin B5 (pantothenic acid) | 7.5 mg |
| Vitamin B6 (pyridoxine HCL) | 6.9 mg |
| Vitamin B12 (cyanocobalamine) | 4.8 mcg |
| Biotin (D-biotin) | 157 mcg |
| Calcium (carbonate & calcium diphosphate) | 250.0 mg |
| Phosphorous (calcium diphosphate) | 50.0 mg |
| Iodine (potassium iodide) | 76 mcg |
| Iron (ferric orthophosphate) | 9.0 mg |
| Magnesium (magnesium oxide) | 104.3 mg |
| Copper (copper gluconate) | 1.0 mg |
| Zinc (zinc oxide) | 10.0 mg |
| Manganese (manganese gluconate) | 2.5 mg |
| Selenium (selenium yeast) | 100 mcg |
| Chromium (chromium yeast) | 100 mcg |
| Molybdenum (molybdenum yeast) | 50 mcg |
| Choline (choline bitartarate) | 12 mcg |
| Ginkgo biloba extract | 60.0 mg |
| Green barley | 400.0 mg |
| Siberian Ginseng extract | 20.0 mg |
| Inositol | 25.0 mg |
| Para-aminobenzoic acid (PABA) | 15.0 mg |
| Juice concentrate | 113.0 mg |
| Kelp | 10.0 mg |

Glutathione reductase (GR) was purchased from Boehringer Mannheim (GmbH, Germany). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA (Richmond, VA). The other chemicals, reagents and enzyme standards were ordered from Sigma Chemical Co. (St. Louis, MO, USA).

The HPLC system (P2000 with AS1000 autosampler) was from Thermo Separation Products (San Jose, CA). Columns with 5 μm $C_{18}$ reversed-phase packing, 250 × 4.60 mm (for vitamin C analysis) and 150 × 4.6 mm (for vitamin E and β-carotene analysis) and 50 × 4.6 mm guard columns with the same packing, were products of Phenomenex (Torrance, CA). Atomic absorption spectrometer (Model 3100) and the flow injection analysis system (FIAS 400) were manufactured by Perkin Elmer Co. (Norwalk, CT). The spectrophotometer (DU 640) was a product of Beckman Coulter, Inc. (Fullerton, CA). The Labconco 25 well rapid digestor, Model 23012, was made by Labconco Co. (Kansas City, MO).

### 2.3. Chemical analyses

Plasma vitamin C was analyzed using the HPLC method developed by Ross [17] with a minor modification to the

sample preparation procedure, while the chromatographic conditions were kept the same. One volume of 30% TCA was added to 5 volumes of plasma and, after centrifugation, the supernatant was mixed with an equal volume of 2× concentrated mobile phase so that the sample injected was in the normal mobile phase and the base line disturbance was minimized.

The protocol for the analysis of plasma vitamin E and β-carotene and RBC vitamin E was a method modified from that of Bieri et al. [18]. Briefly, plasma (0.5 ml) was mixed with 1.5 ml of cold ethanol to denature the protein and then extracted with 2 ml hexane. Alpha-tocopheryl (8–10 μg) acetate was used as an internal standard. The lipid residue in the hexane layer, after drying under $N_2$ flow, was dissolved in 0.5 ml ether/ethanol (1/3, v/v) and then subjected to HPLC analysis. The extraction of RBC suspension followed the same protocol except that the internal standard amount was 1.6–2 μg and the final lipid residue was dissolved in 0.25 ml solvent mix. RBCs had been washed three times with PBS containing 0.5% pyrogallol and finally suspended in the same medium to 50% hematocrit before the extraction. The mobile phase is a gradient mix of two solvents, Solvent A and Solvent B, eluting at 1.0 ml/min. For a volume of 1,000 ml, Solvent A consisted of 1.96 g ammonium acetate, 10 ml water, 600 ml iso-propanol and the volume balance in acetonitrile; Solvent B consisted of 1.96 g ammonium acetate, 250 ml water, 300 ml isopropanol and the rest of the volume in methanol. The gradient program for plasma samples is: 0 min, 0% A; 15 min, 60% A; 35 min, 100% A, 40 min, 100% A; and that for RBC samples is: 0 min, 50% A, 20 min, 90% A.

The method for selenium determination was developed in this lab with reference to Welz and Melcher [19]. Whole blood (0.5 ml), or RBC suspension (0.75 ml) in PBS, 50% hematocrit, was digested with 1 ml $HNO_3$ at 150°C for 1 hr. in a test tube (200 mm × 20 mm i.d.). After cooling the tube to room temperature, 2.0 ml hydrogen peroxide ($H_2O_2$) was added in and the tube was heated again at 150°C for 10 min, and then the temperature was raised to 260°C. The digestion was finished when the residual volume was reduced to about 2 ml. Before going to the FIAS analysis, the sample was heated with 2 ml concentrated HCl for 30 min in a 95°C water bath as the reduction step.

Total glutathione was assayed by an enzymatic recycling procedure in which it is sequentially oxidized by 5,5′-dithiobis(2-nitro-5-thiobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR [20]. The rate of 2-nitro-5-thiobenzoic acid formation was monitored at 412 nm and the glutathione present was evaluated by comparison of that result with a standard curve. Specific measurement of oxidized glutathione (GSSG) was performed after inhibition of the reduced glutathione (GSH) under the action of 2-vinylpyridine, and the GSH concentration was obtained from the difference between total glutathione and the GSSG [21]. Results are expressed as mol/g hemoglobin.

### 2.4. Enzymatic analyses

CAT activity was assessed according to Aebi [22]. This method is based on the decomposition of hydrogen peroxide by CAT. The decomposition rate of $H_2O_2$ was monitored at 240 nm. CAT activity is expressed as U/mg hemoglobin. One CAT unit was defined as the enzyme activity required to consume 1 μmol $H_2O_2$ in 1 min.

GPX activity was determined by the coupled enzyme procedure [23] modified by Whanger et al. [24]. Briefly, GPX catalyzes the oxidation of GSH by *tert*-hydroperoxide. In the presence of GR and NADPH, the GSSG is immediately converted to GSH with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm was measured. One GPX unit was defined as the enzyme activity necessary to convert 1 μmol NADPH to $NADP^+$ in 1 min. The results are represented as U/g hemoglobin.

SOD activity was assayed according to McCord and Fridovich [25] on the basis of the inhibitory effect of SOD on ferricytochrome *c* reduction by $O_2^-$ generated by the action of xanthine oxidase on xanthine. The reduction of ferricytochrome *c* was measured at 550 nm. One SOD unit was defined as the enzyme activity required to inhibit the rate of ferricytochrome *c* reduction by 50% in 1 min.

### 2.5. Erythrocyte susceptibility to free radical oxidation

The erythrocyte susceptibility to oxidative destruction was evaluated *in vitro* by subjecting the cells to oxidative stress which was caused by a free radical initiator, AAPH [26,27]. Oxidative damage was indicated by the extent of hemolysis which was directly resulted from erythrocyte membrane destruction. Briefly, 0.100 ml of whole blood was preincubated in 1.00 ml PBS at 37°C for 5 min. Thereafter, 1.00 ml of 10 mM disodium phosphate ($Na_2HPO_4$) solution containing AAPH (123 mM) was added to the whole blood suspension. The reaction mixture was shaken gently at 37°C under an aerobic condition for three and a half hours. Reaction mixture (0.200 ml) was withdrawn into 2.50 ml of ice cold PBS at 120, 150, 180 and 210 min after AAPH addition and centrifuged at 2000 rpm for 10 min. The extent of hemolysis was measured spectrophotometrically at 540 nm. Similarly, 0.200 ml of reaction mixture was treated with 2.50 ml of distilled water to yield complete hemolysis. Percentage hemolysis was calculated according to the equation: % hemolysis = absorbance of the sample aliquot/absorbance of the complete hemolysis × 100. Data was represented as the time required to achieve 50% hemolysis ($T_{50}$ min).

### 2.6. Statistical analysis

Statistical analysis was performed using StatMost program (DataMost Inc, Salt Lake City, Utah). General student's *t*-tests were used for comparisons between the placebo group and supplement group. Paired student's *t*-tests

Table 2
Effect of multivitamin and multimineral supplementation on plasma vitamin C, E, β-carotene concentration, whole blood selenium concentration and erythrocyte vitamin E concentration of healthy volunteers

| Supplementation Time | −2 week | 0 week | 5 week[a] | 16 week |
|---|---|---|---|---|
| Plasma vitamin C (μmol/L) | | | | |
| Placebo | 40.4 ± 23.7 | 45.1 ± 17.6[b] | 48.5 ± 28.4 | 40.0 ± 19.8 |
| Supplement | 43.5 ± 20.8 | 31.3 ± 22.8[c] | 75.2 ± 37.1*,†† (+55%) | 65.0 ± 32.3*,† (+63%) |
| Plasma vitamin E (μmol/L) | | | | |
| Placebo | 17.4 ± 6.36 | 17.8 ± 4.74 | 18.9 ± 8.36 | 18.5 ± 6.34 |
| Supplement | 19.2 ± 6.48 | 19.0 ± 6.20 | 31.6 ± 12.98**,†† (+67%) | 27.9 ± 7.82**,†† (+51%) |
| Plasma β-carotene (μmol/L) | | | | |
| Placebo | 0.63 ± 0.26 | 0.73 ± 0.32 | 0.84 ± 0.35 | 0.60 ± 0.17 |
| Supplement | 0.75 ± 0.69 | 0.67 ± 0.34 | 2.31 ± 1.55**,†† (+175%) | 1.99 ± 1.53**,†† (+232%) |
| Erythrocyte vitamin E (ng/g Hb) | | | | |
| Placebo | 0.65 ± 0.10 | 0.68 ± 0.08 | 0.62 ± 0.08 | 0.73 ± 0.14† |
| Supplement | 0.67 ± 0.16 | 0.68 ± 0.18 | 1.02 ± 0.24**,†† (+65%) | 1.06 ± 0.25**,†† (+45%) |
| Whole blood selenium (ppb) | | | | |
| Placebo | 114 ± 19.2 | 102 ± 16.1† | 102 ± 12.9† | 109 ± 28.1 |
| Supplement | 110 ± 18.0 | 103 ± 27.0 | 120 ± 16.2**,† (+18%) | 132 ± 37.2*,†† (+21%) |

Data was shown as mean ± SD.
Percentage change from placebo was shown in parentheses.
**,* Significant difference between supplement and placebo: * $P < 0.05$; ** $P < 0.01$.
†,†† Significantly different as compared to baseline (−2 week): † $P < 0.05$; †† $P < 0.01$.
[a] n = 15, [b] n = 14, [c] n = 13.

were used to compare the differences between different time point. $P$ value <0.05 was considered significant. Simple linear regression analysis was used to describe the relationship between variables.

## 3. Results

### 3.1. Chemical antioxidant levels

The effect of multinutrient supplementation on plasma vitamin C, E and β-carotene level, whole blood selenium level and erythrocyte vitamin E level is illustrated by the data in Table 2. Of all five listed categories, the differences between the placebo group and the supplement group at −2 and 0 week were insignificant. The $P$ values between the two groups were from 0.4 to 0.8. The mean plasma vitamin C level of the supplement group was 31% less than that of the placebo group at 0 week, but the difference was not statistically significant ($P = 0.9$). Supplementation with the multinutrient pack, however, significantly increased the chemical antioxidant level of all five categories at 5 week or 16 week of supplementation. Plasma vitamin C, E and erythrocyte vitamin E level all showed an increase approximately in the same range (45–70%), while plasma β-carotene got a two-fold increase, and whole blood selenium only increased about 20%. Of the supplement group, the data of all five categories between 5 and 16 week showed no significant difference. This indicated that the maximum concentration of these chemical antioxidants, at the supplementation dose given above, could be reached in 5 weeks. Continued supplementation did not increase further, but maintained the maximum concentrations.

### 3.2. Activities of antioxidative enzymes

Data on the activities of CAT, GPX and SOD of erythrocyte before and after multinutrient supplementation was presented in Table 3. No significant difference was shown between the supplemented and placebo groups for the three enzyme activities at 5 weeks after supplementation. However, CAT and GPX activities were significantly increased at 16 weeks after supplementation. CAT activity was elevated by 9.9% from 215 ± 17.0 U/mg Hb in the placebo group to 237.0 ± 36.5 U/mg Hb in the supplement group ($P < 0.05$). GPX activity showed significant increase and was markedly higher by 25.7% in the supplemented group (36.09 ± 10.67 U/g Hb) than in the placebo group (28.72 ± 7.32 U/g Hb). At 5 and 16 weeks of supplementation, the SOD activity of the supplement group also showed noticeable increase as compared to the placebo group, but the statistical significance was not high ($P \leq 0.1$).

### 3.3. Erythrocyte glutathione values

GSH concentration was significantly higher by 18.8% in the supplement group than in the placebo group at 16 weeks of supplementation ($P < 0.05$), but not at 5 weeks of supplementation (Table 4). Moreover, although GSH at 5 and 16 weeks was significantly different from that at −2 week, they were not significantly different from that at 0 week. No significant variation was observed in GSSG values between the supplemented and placebo group. In addition, there was a tendency, although not significant ($P = 0.06$), toward an 11% elevation in the GSH to GSSG ratio at 16 weeks of supplementation.

Table 3
Effect of multinutrient supplementation on catalase (CAT), glutathione peroxidase (GPX) and superoxide dismutase (SOD) activities

| Supplementation time | −2 week | 0 week | 5 week[b] | 16 week |
|---|---|---|---|---|
| CAT (U/mg Hb) | | | | |
| Placebo[a] | 210.2 ± 15.3 | 213.5 ± 14.7 | 210.6 ± 21.7 | 215.7 ± 17.0 |
| Supplement | 214.6 ± 21.3 | 209.0 ± 24.3 | 225.5 ± 21.9$^{\dagger}$ (+7.1%) | 237.0 ± 36.5*$^{,\dagger}$ (+9.9%) |
| GPX (U/g Hb) | | | | |
| Placebo[a] | 29.20 ± 4.62 | 28.98 ± 5.86 | 28.58 ± 6.91 | 28.72 ± 7.32 |
| Supplement | 28.23 ± 7.56 | 30.76 ± 7.82$^{\dagger}$ | 30.04 ± 7.00$^{\dagger}$ (+5.1%) | 36.09 ± 10.67*$^{,\dagger\dagger}$ (+25.7%) |
| SOD (U/mg Hb) | | | | |
| Placebo[a] | 2.76 ± 0.34 | 2.75 ± 0.36 | 2.86 ± 0.23 | 2.74 ± 0.33 |
| Supplement | 2.74 ± 0.33 | 2.74 ± 0.29 | 3.09 ± 0.37$^{\dagger}$ (+8.0%) | 2.98 ± 0.47 (+8.8%) |

Data was shown as mean ± SD.
Percentage change from placebo was shown in parentheses.
* Significant difference between supplement and placebo: $P < 0.05$.
$^{\dagger,\dagger\dagger}$ Significantly different as compared to baseline (−2 week): $^{\dagger}$ $P < 0.05$; $^{\dagger\dagger}$ $P < 0.01$.
[a] n = 16, [b] n = 15.

### 3.4. Oxidative hemolysis of erythrocytes

The erythrocyte susceptibility to oxidation was presented as the time required to reach 50% hemolysis ($T_{50}$) and the data were listed in Table 5. The $T_{50}$ was significantly prolonged from 187.7 ± 21.9 minutes in the placebo group to 212.3 ± 30.0 minutes in the supplement group at 5 weeks of supplementation ($P < 0.01$). It was also shown that a significant delayed hemolysis resulted in a 14.2% increase in $T_{50}$ at 16 weeks of supplementation ($P < 0.05$). These results indicate that the erythrocytes are more resistance to AAPH-induced oxidation in healthy people who take multinutrient supplement for 5 weeks and more.

## 4. Discussion

The overall nutritional and physiologic effects of the supplementation of a comprehensive formula of micronutrients, such as the one used in this study, can be quite far-reaching to evaluate, as indicated by the numerous studies which have shown various beneficial effects of supplementation with a single nutrient or a simple combination of nutrients. The present investigation, however, was focused upon the effect on antioxidant defense system, of which the chemical and enzymatic antioxidant components were chosen as indicators. The results clearly show that both the chemical and enzymatic indicators were favorably altered in these healthy subjects by the supplement. Accordingly, the susceptibility of erythrocyte to free radical induced oxidative destruction was significantly reduced, which was a direct indicator of the general enhancement of cells' antioxidative defense capability.

The rate and magnitude of the response to supplementation, however, was different between the chemical and enzymatical indicators. The chemical indicators responded faster and to a larger extent. This is in line with the observation of Postaire *et al* [28]. Their study showed that supply of β-carotene, vitamin E and vitamin C to healthy volunteers involves an increase of singlet oxygen protection of erythrocytes of subjects. This protection appears very quickly after 15 days of supplementation. The present study

Table 4
Reduced glutathione and oxidized glutathione values in healthy people before and after multinutrient supplementation

| Supplementation time | −2 week | 0 week | 5 week[b] | 16 week |
|---|---|---|---|---|
| GSH (μmol/g Hb) | | | | |
| Placebo[a] | 2.78 ± 0.30 | 2.84 ± 0.31 | 2.82 ± 0.54 | 2.77 ± 0.54 |
| Supplement | 2.83 ± 0.29 | 3.04 ± 0.35$^{\dagger\dagger}$ | 3.04 ± 0.35$^{\dagger}$ (+7.8%) | 3.29 ± 0.87*$^{,\dagger}$ (+18.8%) |
| GSSG (μmol/g Hb) | | | | |
| Placebo[a] | 0.218 ± 0.04 | 0.208 ± 0.05 | 0.210 ± 0.06 | 0.227 ± 0.04 |
| Supplement | 0.215 ± 0.04 | 0.205 ± 0.03 | 0.222 ± 0.04 (+5.7%) | 0.243 ± 0.05 (+7.0%) |
| GSH/GSSG | | | | |
| Placebo[a] | 12.75 ± 2.26 | 13.65 ± 3.48 | 13.43 ± 3.27 | 12.20 ± 2.11 |
| Supplement | 13.16 ± 2.61 | 14.83 ± 3.81 | 13.69 ± 2.56 (+1.9%) | 13.54 ± 1.85 (+11.0%) |

Data was shown as mean ± SD.
Percentage change from placebo was shown in parentheses.
* Significant difference between supplement and placebo: $P < 0.05$.
$^{\dagger,\dagger\dagger}$ Significantly different as compared to baseline (−2 week): $^{\dagger}$ $P < 0.05$; $^{\dagger\dagger}$ $P < 0.01$.
[a] n = 16, [b] n = 15.

Table 5
Time required to achieve 50% hemolysis ($T_{50}$, min) before and after multinutrient supplementation

| Supplementation time | −2 week | 0 week | 5 week[b] | 16 week[a] |
|---|---|---|---|---|
| Placebo | 187.5 ± 21.4 | 185.2 ± 23.1 | 187.7 ± 21.9 | 190.3 ± 35.8 |
| Supplement | 191.7 ± 20.5 | 185.6 ± 26.9 | 212.3 ± 30.0**,†† (+13.1%) | 217.3 ± 31.8*,† (+14.2%) |

Data shown as mean ± SD.
Percentage change from placebo was shown in parentheses.
*,** $T_{50}$ significantly different between supplement and placebo: * $P < 0.05$; ** $P < 0.01$.
†,†† Significantly different as compared to baseline (−2 week): † $P < 0.05$; †† $P < 0.001$.
[a] n = 16, [b] n = 15.

also showed that chemical antioxidant arrived the maximum concentration fast (≤5 weeks). Since the antioxidative enzyme activity increase was unable to be observed until 16 weeks of supplementation, the increase of chemical antioxidants, therefore, must be responsible for the $T_{50}$ increase at 5 week.

Antioxidative enzymes cooperate with antioxidants to defend against oxidative stress-induced cellular damage. The first line enzymatic antioxidant is SOD which catalyses dismutation of the superoxide anion ($O_2^{\cdot-}$) into $H_2O_2$, which is then converted to $H_2O$ by CAT and GPX in synergy with GSH. GPX can also reduce organic peroxidase into their corresponding alcohol [29]. Our current results demonstrated that a 16-week supplementation with multinutrient significantly elevated RBC CAT and GPX activities ($P < 0.05$) and slightly raise RBC SOD activity ($P = 0.1$) (Table 3). The delay in the increase in the activities of antioxidant enzymes is probably related to the relative long turnover of RBC. Because enzyme synthesis cannot take place in the mature non-nucleated RBC, the enzyme expression should be regulated during RBC development [30]. The uptake and regulatory events of chemical antioxidants like vitamin E, however, take place mainly, if not only, in the plasma membrane during the whole life of RBCs [31]. For a healthy subject, therefore, an elevated plasma level of chemical antioxidants such as vitamin E would quickly result in an increase of the same in mature erythrocytes, as demonstrated in this study by the observation of simultaneous increase of both plasma and erythrocyte vitamin E in response to the supplementation (Table 2). As an internal part of GPX, it was shown that the higher whole blood selenium concentration, the greater GPX activity [32]. Agree with this point, our data showed that whole blood selenium concentration increased after supplementation (Table 2) and it was strongly correlated to GPX activity ($r = 0.533$; $P < 0.005$ at 5 weeks and $r = 0.408$; $P < 0.05$ at 16 weeks of supplementation).

Previous investigators documented that Vitamin E could influence the enzymatic antioxidants in addition to it acts as a free radical scavenger. Vitamin E supplementation significantly increased erythrocyte CAT activity in both smokers and non-smokers and erythrocyte GPX and GR activities in non-smokers. After supplementation with vitamin E there was a concomitant fall in erythrocyte SOD activity and total glutathione concentration [33]. Vitamin E supplementation to hyperthyroid rats induced a significant decrease in GPX activity and a significant increase in GSH level. And this effect might reduce the burden of oxidative stress in hyperthyroidism [34]. Another study showed that rat fed with vitamin E lead to the elevation of plasma vitamin E level, RBC GSH status, and hepatic cytosolic SOD and GR activities, but has no effect on RBC SOD and hepatic cytosolic GPX activities [35].

For the test of AAPH induced hemolysis, most studies used isolated erythrocyte suspension for the simplicity of the system and, therefore, the data interpretation. Bieri *et al* [18], however, found that very significant loss of erythrocyte vitamin E might occur if an antioxidant (pyrogallol) had not been included in the washing and suspending media of the erythrocytes. Our preliminary tests in this study confirmed their conclusion. Since inclusion of an antioxidant can not be an option for this test, whole blood was employed as the *ex vivo* sample for this study to ensure the erythrocytes were tested in the least modified state. By incorporating plasma into the system, moreover, the antioxidative capacity of plasma, which might be altered by the supplementation, was also included in the investigation as what had been attempted by Abella *et al* [36]. The $T_{50}$ value of this study, therefore, is an overall expression of the antioxidative capacity of the whole blood which was an integration of all the variable factors. The emphasis on fasting blood, however, minimized the influence of diet immediately before sampling.

The method for detection of free radical oxidation used in the present study has advantages in that azo compound decomposes thermally to give radicals without biotransformation of enzyme and the rate and site of radical generation are easily controlled and measured. It was shown that the RBC susceptibility to oxidation is strongly correlated with RBC vitamin E content [27]. In our current report, there was a positive correlation between $T_{50}$ and RBC vitamin E at 5 weeks ($r = 0.181$; $P < 0.05$) and 16 weeks of supplementation ($r = 0.265$; $P < 0.05$). The positive correlation between $T_{50}$ and plasma vitamin E at 5 weeks ($r = 0.382$; $P < 0.05$) was more significant than that at 16 weeks of supplementation ($r = 0.315$; $P < 0.1$).

Peroxidation of RBC membranes resulting in lipid and protein abnormalities which can alter membrane function

and stability. This, in turn, may lead to decreased RBC survival [37,38]. Some antioxidative enzymatic activity decrease in erythrocyte aging [39], this decrement may make the red cells more susceptible to attacks by free radicals. Indeed, membrane lipid peroxidation occurs during the *in vivo* aging of human erythrocytes [40]. In addition, erythrocyte life-span in all mammals is significantly correlated with the levels of SOD, GPX and GSH [41,30]. According to the above reports, the increase in RBC vitamin E and the antioxidative enzymatic activities shown in our results might not only lead to the reduction in the susceptibility of RBC to AAPH-induced hemolysis but probably also can cause prolonged RBC survival.

In conclusion, the present study of healthy subjects showed that short-term supplementation (16 weeks) with multinutrient can beneficially modify the antioxidant defense system, as indicated by the improved antioxidant vitamin status and elevated antioxidant enzymatic activities. These improvements resulted in the reduction of RBC susceptibility to free radical peroxidation.

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