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Effects of Antioxidant Supplementation on Antioxidant Status and PHA-Stimulated Interleukin-2 Production by Peripheral Blood Mononuclear Cells in the Elderly Women*

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ABSTRACT

This study was done to investigate effects of antioxidant supplementation on phytohemagglutinin (PHA)-stimulated interleukin-2 (IL-2) production by peripheral blood mononuclear cells (PBMCs) in elderly women. This study was designed as a placebo-controlled, single-blinded, randomized intervention trial. Twenty four elderly women aged over 60 years, visitings social welfare center in Seoul were divided into 3 groups, placebo (n = 8), vitamin C supplemented (n = 8), and vitamin E supplemented (n = 8) groups. Experimental groups were given either 1000mg of L-ascorbic acid or 400 IU of d- α-tocopherol for 4 weeks. There was no significant difference in antioxidant vitamins intakes and their plasma levels among pre-intervention groups. Plasma viatmin C or E levels was significantly increased after vitamin C or E supplementations. The increases of plasma thiobarbituric acid-reactive substance (TBARS) levels in the placebo group were significantly higher than those of the supplemented 2 groups. There were no significant differences in the changes of plasma IL-2 level between pre- and post-intervention among the 3 groups. However there was a significant increase in PHA-stimulated IL-2 production by PBMCs after 4-week vitamin E or vitamin C supplementation. Particularly, vitamin E supplemented group showed a higher PHA-stimulated IL-2 production than vitamin C supplemented group. These results indicate that vitamin E or vitamin C supplementation might enhance mitogen-stimulated cytokine production by immune cells, which could be one of the factors to improve health status in the elderly. (*J Community Nutrition* 7(1) : 42~48, 2005)

KEY WORDS : antioxidant supplementation · antioxidant status · IL-2 · PBMC · elderly women.

Introduction

Aging has been associated with a decline in T-cell mediated immunity, including mitogen-induced T-cell proliferation, delayed-type hypersensitivity (DTH) and interleukin-2 (IL-2) production (Hallgren et al. 1983 ; Meydani 1999). IL-2 is known to be an essential cytokine for the stimulation of immune cells, particularly T-cell. *In vitro* IL-2 production by peripheral blood mononuclear cells (PBMCs) was commonly assessed to evaluate T-cell reactivity.

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The decline in the cellular immune responsiveness such as T-cell mediated immunity in the elderly is generally associated with an increased incidence of infectious, neoplastic, and autoimmune diseases (Cakman et al. 1996). Therefore, an effective method to prevent or delay this age-related impairment of protective immune function would be of great public health significance, particularly for elderly persons.

Antioxidant vitamins play an important role to maintain optimal immune function since the depressed immune responsiveness observed in the elderly has been associated with an increase in free radical (Hatman, Kayden 1979 ; Meydani et al. 1995). Oxidative stress resulted in suppression of IL-2 production. Many studies suggest that in a population under immunologic stress, supplementation of antioxidant vitamins may be beneficial (Bergsten et al. 1990 ; Meydani et al. 1995)

Antioxidants, such as vitamin E, vitamin C and β-carotene enhance immune function when administered supplementally to animals or to culture systems *in vitro* Most results were

obtained from animal models (Adolfsson et al 2001 ; Serafini 2000 ; Wakikawa et al. 1999) and cell culture (Calder, Newholme 1993 ; Lee et al. 1993). Some human studies were performed with elderly humans (Meydani et al. 1997 ; Meydani et al. 1998). Evaluation has been carried out by DTH response (Meydani et al. 1990 ; Meydani et al. 1997) or on humoral immunity such as levels of circulationg IgG or IgM antibodies (Tanaka et al. 1979). One mechanism for immunostimulatory effects of these antioxidants is the ability to modulate the production of cytokines that regulate immune function (Han, Meydani 2000).

In spite of the increasing population of elderly, there are limited reports about antioxidant vitamins supplementation and its relationship to immune response of the elderly in Korea. Kim (1999) reported that vitamin E supplementation at 400IU/day for 4 weeks increased significantly plasma vitamin E concentration and decreased TBARS levels. In addition, it restored the percentage of neutrophils, lymphocytes, and eosinophils which had been out of normal ranges before supplementation and increased helper T-cell percentage. They suggest that moderate vitamin E supplementation can improve immune response, especially nonspecific immunity and cell mediated immunity via protection of oxidant stress. But they did not examined cytokine production by PBMCs with vitamin supplementation.

This study was done to examine the effects of antioxidant vitamins supplementation on the improvement of immune response by measuring mitogen-stimulated IL-2 production by PBMCs.

Subjects and Methods

1. Subjects and study design

This study was designed as a placebo-controlled, singleblinded, randomized intervention trial. Subjects were 24 elderly women aged over 60 years, visiting Jangwhi Social Welfare Center of Seongbook-Gu in Seoul. The elderly women were divided into 3 groups, placebo (n = 8), Vit C supplemented (n = 8), and Vit E supplemented (n = 8) groups. Experimental groups were given either 1000mg of L-ascorbic acid (Roche Ltd) or 400IU (287mg) of d- α-tocopherol (grandpherol, Yuhan Co) for 4 weeks. Placebo group was given an empty capsule. The subjects were allowed to eat vitamin supplements after lunch every day. Dietary assessment and biochemical analysis were performed for 20 subjects excluding 4 subjects quitting the supplementation during the intervention period.

2. Dietary assessment

Dietary data was obtained through questionnaires by interviewers trained for nutritional survey. Dietary intakes were assessed by semi-quantitative food frequency questionnaires (SFFQ) that include 98 commonly consumed food items selected from the Korean National Health and Nutrition Survey for the elderly population and were verified validity by Lee et al. (2002). Nutrient intakes were analyzed by SFFQ computer program developed by our team.

3. Plasma antioxidant vitamins and TBARS

Blood collection was taken before and after the 4-week intervention period. Venous blood was obtained by using 10-ml vacutainers containing heparin. Blood was centrifuged to separate plasma to determine antioxidant vitamins. Vitamin C was assayed from plasma samples pretreated with 0.75M metaphosphoric acid by 2, 4-dinitrophenylhydrazine method (Pesce, Kaplan 1987) using a UV spectrophotometer (Uvikon 930, Kontron, Switzerland).

Vitamins A and E were assessed by measuring retinol and α-tocopherol, respectively (Bieri et al. 1979). Plasma vitamins A and E were extracted with ethyl alcohol and hexane. Retinol and -tocopherol were separated by HPLC (712 HPLC System, Gilson Medical Electronics, France) on Nova-Pak C18 (3.9 × 150mm, Waters, Ireland) column using methanol-water (95 : 5, v/v) as the mobile phase. Elution was detected spectrophotometrically at 292nm.

Plasma β-carotene was extracted with absolute alcohol : distilled water : hexane (1 : 1 : 2) and separated by HPLC on Nova-Pak C18 (3.9 × 150mm, Waters, Ireland) column using acetonitrile : dichloromethane : methanol (7 : 2 : 1) as mobile phase. Elution was detected spectrophotometrically at 452nm (Bieri et al. 1985).

Plasma lipid peroxide level was assayed by TBARS method (Ohkawa et al. 1979).

4. IL-2 production by PBMCs

1) PBMCs separation

Venous blood was obtained by using 10-mL Vacutainers containing heparin.

PBMCs were isolated from the four 10-mL evacuated tubes by density gradient centrifugation with Histopague within 2h after venipuncture. The cells at the plasma Lymphoprep interface were harvested, washed twice in complete RPMI

medium 1640 with 10% (by vol) heat-inactivated fetal calf serum and penicillin and streptomycin.

2) IL-2 production by PBMCs

IL-2 production by PBMCs pre- and post-intervention were measured simultaneously after 40 h of stimulation with 0mg/L 3mg/L concentrations of purified PHA. PBMC was thawed and washed twice with complete RPMI medium 1640 and 2.0×10^6cells (IL-2), 4.0×10^4cells (IL-6) were cultured in 1mL complete RPMI containing 0 or 3mg PHA/L in 24-well culture plates. Viability of the cells, as evaluated by trypan blue exclusion, always exceeded 90%. After incubation (40h at 37℃ in 5% CO_2), the plates were centrifuged for 10 min at 650 × g. Supernates were collected and cytokine production was measured by ELISA using commercial kits.

Table 1. Comparison of general characteristics in elderly women before intervention

Variables	Placebo	Vit.C suppl	Vit.E suppl
Age (year)	76.0 ± 5.8^{NS}	75.9 ± 9.8	73.9 ± 8.5
Height (cm)	146.7 ± 3.1	146.7 ± 5.7	146.0 ± 8.3
Weight (kg)	54.8 ± 3.1	53.5 ± 11.1	52.2 ± 10.0
Muscle	32.6 ± 2.0	31.3 ± 4.1	31.9 ± 5.4
%fat	36.6 ± 2.4	36.7 ± 6.6	34.6 ± 3.9

NS : not significantly different among groups

5. Statistical analysis

All data were expressed as mean ± SD. Statistical analy-

Table 2. Comparison of nutrient intakes and % RDA in elderly women

Variables	Placebo		Vit.C suppl		Vit.E suppl	
Energy (kcal)	1178.7 ± 491.2	71.3 (66.7)[1)]	1258.6 ± 621.8	75.3 (57.1)	1248.0 ± 229.6	74.7 (28.6)
Protein (g)	37.2 ± 18.4	67.6 (66.7)	42.9 ± 22.8	77.9 (42.9)	45.0 ± 11.5	81.8 (42.9)
Ca (mg)	228.8 ± 152.5	32.7 (100)	351.8 ± 229.6	50.3 (85.7)	352.3 ± 146.0	50.3 (85.7)
P (mg)	617.2 ± 312.8	88.2 (50.0)	707.4 ± 350.2	101.1 (14.3)	741.7 ± 200.8	106.0 (0.0)
Fe (mg)	6.8 ± 3.3	56.2 (83.3)	7.5 ± 4.6	62.7 (71.4)	8.0 ± 2.6	66.5 (71.4)
Vit A (ugRE)	313.0 ± 187.0	44.7 (83.3)	377.6 ± 275.5	53.9 (85.7)	367.3 ± 110.0	52.5 (85.7)
Retinol (ug)	40.1 ± 39.6		57.5 ± 53.6		71.9 ± 39.8	
β-carotene (ug)	1571.3 ± 886.4		1852.4 ± 1391.7		1689.5 ± 545.1	
Thiamin (mg)	0.7 ± 0.3	73.3 (50.0)	0.8 ± 0.4	81.6 (42.9)	0.7 ± 0.2	73.0 (42.9)
Riboflavin (mg)	0.7 ± 0.5	60.2 (66.7)	0.8 ± 0.4	64.6 (71.4)	0.8 ± 0.2	69.8 (71.4)
Niacin (mgNE)	9.1 ± 4.7	70.1 (66.7)	9.3 ± 4.5	71.7 (57.1)	9.7 ± 2.6	74.3 (71.4)
Vit C (mg)	64.7 ± 36.2	92.4 (33.3)	83.4 ± 87.3	119.1 (42.9)	57.0 ± 23.4	81.5 (71.4)

Values are mean ± SD, 1) % RDA, ( ) : % of subjects consumed below 75% of RDA

Table 3. Changes of plasma antioxidant status between pre- and post-intervention in elderly women

Variables		Placebo	Vit.C suppl	Vit.E suppl
Vitamin C (mg/L)	Pre	6.12 ± 3.49	6.31 ± 4.06	6.03 ± 2.94
	Post	11.87 ± 4.89**	17.50 ± 4.11***	14.39 ± 3.74***
	Change	5.75 ± 2.80^b	11.37 ± 3.68^a	8.35 ± 1.37^{ab}
Vitamin E (mg/L)	Pre	6.16 ± 1.28	5.99 ± 1.47	6.01 ± 2.30
	Post	5.67 ± 1.46	8.60 ± 5.18	11.69 ± 6.03
	Change	−0.49 ± 1.29	2.61 ± 5.45	5.68 ± 7.71
Vitamin E (mg/g TG1 + Chol)	Pre	8.41 ± 0.90	8.05 ± 1.72	7.89 ± 3.28
	Post	7.51 ± 1.93	11.69 ± 5.12	14.91 ± 6.30*
	Change	−0.90 ± 1.24^b	3.64 ± 6.07^{ab}	7.02 ± 7.59^a
Vitamin A (mg/L)	Pre	0.49 ± 0.07	0.43 ± 0.14	0.47 ± 0.16
	Post	0.55 ± 0.14	0.49 ± 0.08	0.57 ± 0.23
	Change	0.07 ± 0.16	0.06 ± 0.20	0.93 ± 2.19
β-carotene (mg/L)	Pre	0.10 ± 0.04	0.15 ± 0.10	0.11 ± 0.05
	Post	0.13 ± 0.06	0.13 ± 0.07	0.12 ± 0.06
	Change	0.02 ± 0.04	−0.02 ± 0.08	0.01 ± 0.04
TBARS (mg/L)	Pre	2.52 ± 1.23	5.02 ± 2.38	2.76 ± 1.18
	Post	5.74 ± 2.19	5.06 ± 1.63	4.73 ± 2.14
	Change	3.23 ± 1.04^a	0.04 ± 3.25^c	1.98 ± 2.12^b

*, **, *** : significantly different between pre- and post- intervention at $p<0.05$, $p<0.01$, $p<0.001$ by paired t-test
a, b, ab : means with different superscript letter are significantly different among groups at $p<0.05$ by Duncan's multiple range test

sis was performed by SAS-PC program. Statistical significant difference was determined by using ANOVA, paired student's t-test, Duncan's multiple range test.

Results

1. Baseline characteristics of subjects

There was no significant differences in age and anthropometric measurement among the 3 groups. Therefore the 3 groups appeared to be quite similar as a result of the randomized procedure (Table 1).

2. Daily intakes of antioxidant vitamins of elderly women

The average intakes of vitamin C were 64mg (92.4% of RDA), 83.4mg (119.1%) and 57.0 mg (81.5%) in placebo, Vit C and Vit E supplemented groups, respectively (Table 2). The average intakes of vitamin A were 313.0 μgRE (44.7% of RDA), 377.6 μgRE (53.9%) and 367.3 μgRE (52.5%) in placebo, Vit C and Vit E supplemented groups, respectively The percentage of subjects consumed less than 75% of Korean RDA were about 50% in vitamin C and 85% in vitamin A intakes. There were no significant differences in vitamin C and A intakes among the 3 groups.

3. Plasma levels of antioxidant vitamins and TBARS

There was no significant difference in baseline plasma levels of antioxidant vitamins among the 3 groups (Table 3). However baseline plasma TBARS level of the Vit C supplemented group seemed to be slightly higher than the other groups. Compared to baseline data, there was a significant increase in plasma vitamin C level within all the 3 groups after 4 week of intervention. This data might mean that all the subjects might eat more foods such as oranges or Gimchi, which is a good source of vitamin C during intervention since this study was done in the winter. However the increase of plasma vitamin C level was significantly greater in the vitamin C supplemented group compared to the placebo group.

But there was no significant increase of plasma vitamin E level after 4-week vitamin E intervention at 400IU (287mg/day). However when the unit of plasma vitamin E level was changed into mg per total cholesterol + TG (g), plasma vitamin E levels significantly increased after Vit E supplemented group. The plasma TBARS levels were not significantly lower after supplementation. However the changes of plasma TBARS levels between pre- and post-intervention were significantly lower in the supplemented groups compared to the placebo group.

4. Plasma IL-2 level and IL-2 production by PBMCs

Plasma IL-2 level was unexpectedly significantly increased in the placebo group while the other 2 groups were not changed after 4-week intervention. However there was no significant difference in the changes between pre- and post-intervention among the 3 groups (Table 4).

There was no significant difference in unstimulated IL-2 production by PBMCs after 4 week intervention. However there was a significant increase in PHA-stimulated IL-2 production by PBMCs after 4-week vitamin E or vitamin C supplementation (Table 5). Particularly, the Vit E supplemented group showed a higher PHA-stimulated IL-2 production than the Vit C supplemented group. But these increases did not significantly differ among the 3 groups. Such a lack of significant difference among the 3 groups might be due to being few of number of subjects in each group.

Discussion

This study showed that baseline data for average age and

Table 4. Changes of plasma cytokine levels after 4-week intervention in elderly women

Variables		Placebo	Vit.C suppl	Vit.E suppl
Plasma IL-2 (pg/ml)	Pre	33.0 ± 9.1	40.0 ± 18.0	44.5 ± 16.3
	Post	49.1 ± 15.9*	52.4 ± 10.7	53.2 ± 5.4
	Change	16.0 ± 13.1^{NS}	12.4 ± 21.0	8.7 ± 13.2

* : significantly different between pre- and post- intervention at $p < 0.05$ by paired t-test
NS : not significantly different among groups

Table 5. Changes in PHA-stimulated cytokines production by PBMCs between pre- and post-intervention in elderly women

variables		Placebo		Vit.C suppl		Vit.E suppl	
		PHA (0)	PHA (3)	PHA (0)	PHA (3)	PHA (0)	PHA (3)
IL-2	Pre	19.4 ± 12.3	17.1 ± 29.5	45.5 ± 22.9	0.5 ± 10.5	39.7 ± 36.3	−0.5 ± 12.8
	Post	35.2 ± 25.8	107.6 ± 80.1	33.6 ± 30.0	179.9 ± 86.0*	37.9 ± 26.1	257.2 ± 275.1*
	Change	15.8 ± 24.7^{NS}	90.5 ± 98.3^{NS}	−11.9 ± 18.8	179.4 ± 83.8	−1.8 ± 27.2	257.7 ± 267.1

* : significantly different between pre- and post- intervention at $p < 0.05$ by paired t-test
NS : not significantly different among groups

anthrophometric measurement was the same among 3 different groups. In addition, there was no significant difference in antioxidant vitamins intakes and their plasma levels at baseline. There was a three fold increase of plasma vitamin C level after 4-week vitamin C intervention at 1000mg/day. But there was no significant increase of plasma vitamin E level after 4-week vitamin E intervention at 400IU (287mg) /day. However when the unit of plasma vitamin E level was changed into mg per total cholesterol + TG (g), plasma vitamin E levels two-fold increased after Vit E supplemented group compared to baseline level. Similar to our study, another Chinese study (Lee, Wan 2000) showed that plasma vitamin E level almost doubled after 233 mg/d dl- α -tocopherol supplementation for 28 days compared to baseline level. In accordance with increased plasma Vit E level, plasma malondialdehyde and urinary adduct 8-OH-2'-deoxyguanosine levels as indicators of oxidative stress were significantly decreased. Another study with healthy Korean elderly women also showed that vitamin E supplementation at 400IU/day for 4 weeks increase plasma vitamin E concentration and decrease TBARS levels (Kim 1999). In this study, the plasma TBARS levels were not significantly lower after supplementation. However the changes of plasma TBARS levels between pre- and post-intervention were significantly lower in the supplemented groups than in the placebo group. It is hard to explain the difference of results between this study and Kim study (1999) in terms of plasma TBARS level. In general, plasma TBARS level has known to be not a good and sensitive indicator of oxidative stress. However a lot of studies measure plasma TBARS level as one of indicators of oxidative stress since it is easy to measure compared to other indicators such as urinary adduct 8-OH-2'-deoxyguanosine level or plasma protein carbonyl or F2 total and 8-isoprostanes measured in other studies (Lee, Wan 2000 ; Jacob et al. 2003).

Cytokines are central to immunoregulation, cellular interaction and immune function. The cytokine IL-2 is an important mediator required for T lymphocyte proliferation. Mitogen-stimulated Il-2 production declines with age, subsequently affecting T lymphocyte mediation (Meydani et al. 1998). In this study there was no significant difference in plasma IL-2 level but there was a significant increase in PHA-stimulated IL-2 production by PBMCs after 4-week vitamin E supplementation. Similar to our study, vitamin E supplementation at 400 or 800 IU/day for 30 days increased lymphocyte proliferation and interleukin-2 production and reduced serum lipid hydroperoxides in vitamin-deficient elderly populations (Meydani et al. 1990). Lee, Wan (2000) also reported that vitamin E supplementation at 233 mg/d dl- α -tocopherol for 28 days increased PHA- or lipopolysaccharide (LPS) -induced total-T and T-helper cell proliferation and improved oxidative stress by decreasing hydroperoxide production in lymphocytes in Chinese men and women. Another Korean study with elderly women showed that vitamin E supplementation at 400 IU/d for 4-weeks significantly increased in helper T-cell percentage (Kim 1999). In healthy men, supplementation of vitamin E at 200mg/day for 8weeks helped to prevent oxidative stress induced by dietary polyunsaturated fatty acid as well as to restore lympocyte proliferative response to concanavalin (Kramer et al. 1991). These results indicated that moderate or megadose of vitamin E supplementation improved immune responses, especially cell-mediated immunity via protection of antioxidant stress.

On the other hand, Pallast et al. (1999) investigated whether 3- or 6-month supplementation with low dose of vitamin E (50 or 100mg/day) in the healthy Dutch elderly. They reported that both DTH and IL-2 production showed a trend toward increased responsiveness with increasing dose of vitamin E. 100mg/d of vitamin E supplementation showed more pronounced beneficial effects in cellular immune function.

Little is known about vitamin E modulation of cytokine production. The oxidant-antioxidant balance of vitamin E may be required for the cellular proliferation of T cells in healthy individuals. It has been suggested that vitamin E, via a reduction of prostaglandin E2 (PGE2) production, could stimulate Th1-like response (Meydani 1995). In addition, the mechanism by which vitamin E influenced cell proliferation may be related to its ability to quench free radicals formed as mitogen-induced products of the lipoxygenase pathway, derived from arachidonate metabolism and phospholipid turnover (Meydani 1995 ; Serafini 2000).

Ascorbic acid is the most important soluble antioxidant as well as a cofactor in many hydroxylating reaction. There is a lot of evidence that the immune system is sensitive to the level of ascorbic acid. Lee et al. (1993) reported that culture supernatants of ascorbic acid-treated thymocytes and T-cells enhanced the phagocytic activity of macrophages. In addition, ascorbic acid increased antibody production of splenocytes or B cells *in vitro*. Schwage, Schulze (1998) found that cellular ascorbic acid influence mitogen-induced IL-2 production and mitogen-induced IL-6 production was also affected by prolong

dietary ascorbic acid depletion in deletion-repletion study of pigs. They suggest that ascorbic acid levels exert an early effect on immune homeostasis via reactive oxygen intermediates (ROI)-dependent expression of IL genes since the transcription factor NF-κB is sensitive to ROI and regulates the expression of interleukin genes.

In this study with elderly women, vitamin C supplementation at 1000mg/day for 4 weeks showed no significant difference in plasma IL-2 level but a significant increase in PHA-stimulated IL-2 production by PBMCs although vitamin E supplementation showed a higher PHA-stimulated IL-2 production. Another study showed vitamin C supplementation at 1000mg/day for 4 weeks significantly increased plasma IL-2 level in male college nonsmokers and increased NK cell percentage in male college smokers and nonsmokers (Kim 1998). Jeng et al. (1996) examined the combined effects of vitamin C (1000mg/d) and vitamin E (400mg dl-α-tocopheryl acetate) supplementation for 4 weeks on cytokine production of healthy adults. They found that combined supplementation increased more production of IL-1β and tumor necrosis factor-α (TNF-α) compared to vitamin supplementation alone. This means that combined supplementation of antioxidant vitamins is more immunopotentiating than supplementation with either vitamin alone in healthy adults. Unfortunately in our study we could not investigate the combined effects of vitamins C and E supplementation because we could not handle more subjects than 24 elderly women for PBMC separation and PBMC incubation at the same time.

In conclusion, although the study was carried out on peripheral blood mononuclear cells that represent a restricted compartment of the immune system, overall data suggests that vitamin E or vitamin C supplementation might enhance mitogen-stimulated cytokine production by immune cells, which could be one of the factors to improve health status in the elderly. However further study is needed to clarify the beneficial effects of antioxidant vitamin supplementation with more subjects or synergic effects of several antioxidant vitamins supplementation on immune response for the elderly in the future.

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