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ANTIOXIDANT MICRONUTRIENTS IMPROVE INTRINSIC AND UV-INDUCED APOPTOSIS OF HUMAN LYMPHOCYTES PARTICULARLY IN ELDERLY PEOPLE

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Abstract: *Objective:* Aging and oxidative stress may lead to enhanced cellular damage and programmed cell death.to study the association of intrinsic apoptosis with age and the effect of antioxidant supplementation on intrinsic and UV-induced apoptosis in children, young and elderly people. *Methods:* The study was a 2 months, double-blind, randomized trial. Three age groups were studied: children, young adults and elderly people. A total of 274 healthy subjects were allocated to a group supplemented with moderate amounts of retinol, β-carotene, α-tocopherol, ascorbic acid and selenium or placebo. Plasma oxidative stress parameters were detected and apoptosis of lymphocytes was evaluated with TUNEL staining. *Results:* At baseline, percentages of intrinsic apoptosis were 13.8% and 11.1% in elderly and young people, respectively, both significantly higher than children (6.3%). A decrease of 1.7% and 2.3% in intrinsic apoptosis of lymphocytes was found in the supplemented groups of young and elderly people compared with their control groups (all p values <0.001), but no significant decrease in children. Moreover, percentages UV-induced apoptosis significantly decreased by 1.4%, 1.9% and 3.1% in children, young and elderly people, respectively, compared with control groups after the trial. There were considerable increments in concentrations of plasma β-carotene, retinol, tocopherol, ascorbic acid and selenium in all three treated groups after the supplementation. *Conclusions:* Young and elderly people have a higher intrinsic apoptosis than children, which was improved by antioxidant supplementation. UV-induced damage was attenuated by the supplementation in all three age groups.

Key words: Apoptosis, micronutrient, aging, oxidative stress, UV-radiation.

Backgroud

Peripheral blood lymphocytes consist of both proliferating and apoptotic cells. Apoptosis is a genetically programmed mechanism of cell death, which plays a critical role in the immune function of the body (1), and in various age-related degenerative diseases (2-6). The lymphocyte population of premature newborns may consist of 4.6% of proliferating and 22.1% apoptotic cells (7). Increased apoptosis of peripheral blood lymphocytes is observed in children with nephritic syndrome (8). However, the association of age with the percentage of lymphocyte apoptosis is not clear.

Over recent years, evidence has been accumulating in favor of the free radical theory. Oxidative stress increases with aging and leads to enhanced cellular damage, such as lipid peroxidation, protein oxidation, DNA damage, etc (9). In addition, reactive oxygen species (ROS) can trigger cells to undergo programmed cell death. Increased oxidative stress and decreased ability to cope with ROS could amplify apoptotic cell death in aging lymphocytes as a marker for other cells in the body (10). Ongoing trends in the field are recognition of undetermined oxidant/antioxidant interactions and elucidation of important signaling networks in radical metabolism (11). Therefore, the purpose of this study was to reveal possible differences of age-associated spontaneous apoptosis, and to detect effects of antioxidant supplementation (vitamins E, A, C, β-carotene and selenium) on micronutrient status, and spontaneous and UV-induced apoptosis in children, young and elderly people. It was hypothesized that this antioxidant supply would attenuate lymphocyte apoptosis.

Subjects and Methods

Subjects

This study design was a 2 months, intervention trial. A total of 274 healthy persons from Shandong province, China, comprising 88 children (age 8-10 y), 90 young college students (age 18-22 y) and 95 elderly people (age 60-75y) were recruited. They were randomly allocated to a placebo or supplement condition for each age group in the order of enrollment. Groups C1 (n=48), Y1 (n=45) and O1 (n=50) were placebo groups for children, young and old people, respectively; group C2 (n=40) was supplemented daily with 2000 IU retinol, 1.0 mg β-carotene, 100 mg α-tocopherol (dl-a-tocopheryl acetate), 300 mg ascorbic acid and 200 μg selenium; groups Y2 (n=45) and O2 (n=45) were supplemented with 3000 IU retinol, 1.5 mg β-carotene, 200 mg α-tocopherol, 500 mg ascorbic acid and 400 μg selenium. The capsules were labeled in red, yellow and blue color and manufactured by Hurun company (a Chinese food-additive company, Beijing). Trial participants and the research team were unaware of the treatment assignment. The trial was deblinded after analysis of the primary outcomes. The subject characteristics are shown in Table 1.

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Table 1
Comparison of subject characteristics and indicators among children, younger and old people

Items	Children (mean± SD)	Young people (mean± SD)	Old people (mean± SD)	P[1]
Characteristics				
No of subjects	88	90	95	
Age (years)	10.7±0.7	18.6±1.0	65.3±8.1	0.000
Height (cm)	138.6±7.0	168.4±8.6	161.6±7.9	0.000
Weight (kg)	32.6±5.9	60.0±9.5	62.8±9.3	0.000
BMI (kg/m^2) [2]	--	21.1±2.4[b]	24.0±3.2	0.000
Percentage of apoptosis in lymphocytes				
Intrinsic apoptosis (%)	6.3±1.6 [ab]	11.1±1.4 [a]	13.8±1.6	0.000
UV-induced apoptosis (%)	19.1±2.7[ab]	24.3±1.8 [a]	32.8±2.5	0.000

[1]P: ANOVA analysis; 2BMI indexes of Children did not be calculated, because the formula is not suitable for children; [a], compared with old people; [b], compared with young people; p values < 0.05.

Written, informed consent was obtained from participants or their parents in case of children, prior to entering the trial. Subjects underwent a clinical examination to determine suitability for participation in the study. Exclusion criteria were severe illness; any history of gastro-intestinal surgery or chronic bowel disease; current metabolic illness, such as diabetes mellitus, untreated hyper- or hypothyroidism or severe lipid metabolism disorder; regular intake of nutritional supplements (i.e. vitamin/mineral supplements or micronutrient-enriched foods), unless discontinued for at least four weeks before study entry and throughout the investigation. After ascertainment of eligibility, subjects were enrolled in the study, had a baseline interview and started with their allocated treatments to be taken daily for a period of two months. Subjects were examined or visited once each week by teachers or co-investigators to replenish supplements and to monitor compliance by counting and recording the number of supplements that were taken.

The study was approved by the ethical review committees of Medical College of Qingdao University. Written consent was given by each subject at the start of the trial.

Blood Sample Treatment

Blood samples were taken from all subjects at baseline and the end of the trial, and plasma levels of the micronutrients, -carotene, retinol, vitamin E, vitamin C and selenium were measured. All blood samples were processed immediately for storage. Specimens were stored in polypropylene vials at -80°C for up to 3 months before batched analysis. It has been determined that vitamins C, retinol, -carotene, vitamin E and selenium are stable under these storage conditions for up to 2 years (12).

Micronutrient concentrations in plasma

Retinol, carotenoids and tocopherols were determined in plasma samples according to the method of Hess et al (13). Analyses were performed in a highperformance liquid chromatographic system equipped with an LC-10AD pump. Retinol, carotenoids, and tocopherols were measured at 325, 450, and 292 nm, respectively. A standard curve for each analyte was constructed from authentic standards (Sigma, St. Louis, MO, USA). Standard concentrations were calculated on the basis of their known extinction coefficients. Vitamin C concentrations in subject plasma were determined spectrophotometrically using the 2, 4-dinitrophenylhydrazine (DNPH) method. Ascorbic acid was converted to dehydroascorbic acid in the presence of thiourea and copper sulfate. Dehydroascorbic acid was then coupled with 2,4-DNPH in 9.0 mol/L sulfuric acid to form a bis-2,4-DNPH derivative. When treated with 65% sulfuric acid, this derivative yields a stable brownish-red color that was measured with a spectrophotometer at 520 nm (14-15). Plasma selenium levels were measured using a standardized fluorometric method (16). The usable range is 0.02 to 10 ppm or greater (4 ng detection limit); intra-assay and inter-assay coefficients of variance were 1.5% and 4.2%, respectively.

Apoptosis detection

Peripheral blood lymphocytes (PBL) were isolated from venous blood by Ficoll density gradient centrifugation (3K30 Sigma, Germany) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, glutamine, and penicillin/ streptomycin in a 5% CO2 atmosphere. PBL adjusted at 1×10 6 cells/ml were incubated for 24 h at 37°C. To induce apoptosis, the cells in uncovered dishes were exposed to UVB radiation. The exposure time of 11 min and a distance of 4 cm from the source (20W/12 lamp emitting 2.1 W at 257 nm) produced the maximum percentage of apoptotic cells detected by microscope (17-18).

Apoptosis was detected by TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) assay (19) with accurate quantitation of apoptosis based on nuclear condensation, nuclear fragmentation (20), using a detection kit from Boster company (In Situ Cell Apoptosis Detection kit, Boster Biotechnology Company, Wuhan, China). All the procedures were performed according to the manufacturer's instruction (Boshide Comp Lit.d, Wuhan, China). Positive staining for cell apoptosis presented as brown, located in the nucleus. To define the distribution of apoptosis within each smear, a direct cell count was performed on TUNEL sections using a microscope to count the number of TUNEL-positive cells in 10 microscope fields (12,800 mm^2) per subject at a magnification of 400×(6). Results are expressed as apoptotic cell area/total cell area in percentage.

Statistical analysis

Continuous data are presented as mean ± SD. Baseline variables were compared across treatment groups. The mean differences in change over the intervention period between intervention groups and control group and 95% confidence intervals (CI) were estimated for β-carotene, retinol, α-tocopherol, ascorbic acid and selenium levels and lymphocyte apoptosis by using a general linear model ANOVA. The

apoptotic index (percentage of TUNEL-positive cells) was determined from cell counts of 200–300 total cells in randomly selected fields (400×); the average apoptotic index (mean±SE) was then calculated from the individual counts (21). A $P < 0.05$ was considered as a significance level for all tests.

Results

General characteristics and spontaneous apoptosis of lymphocytes in children, young and elderly people are shown in Table 1. Average plasma levels were well over the cutoff values for deficiencies (0.7 mol/L for retinol, 11.6 mol/L for tocopherol and 11.4 mol/L for ascorbic acid, respectively); plasma level of Vitamin C in children was 30.9 mol/L and significantly higher than young (27.9 mol/L) and elderly (24.6 mol/L) people. At baseline, percentages of spontaneous apoptosis were associated with age, being 13.9%, 11.1% and 6.3% in elderly, young people and children, respectively.

In the intervention study, complete data were available on 93.1% of the original number of 274 subjects, being 83 children, 84 young and 88 old people. For 19 subject blood and plasma volumes were insufficient for the measurement of micronutrient concentrations and lymphocyte collection. There were no substantial differences in any of the baseline characteristics between the placebo and supplemented groups in children, young and old people, respectively (Table 2).

After the 2 months supplementation, there were significant increases of plasma β-carotene, retinol, tocopherol, ascorbic acid and selenium concentrations in all three supplemented groups compared with baseline and compared with the respective placebo groups. Percentages of increase were 20.2% ~36.0% for -carotene (all p values <0.001); 32.3% ~ 47.5% for retinol (all p values <0.001), 15.9% ~ 27.8% for tocopherol (all p values <0.001), and 30.4% ~ 98.6% for ascorbic acid (all p values <0.001) in treated groups of three age population; as was a range of the increases in plasma selenium from 19.6% to 54.4% (all p values <0.05) , respectively compared with the placebo groups (Table 3).

After the trial, apoptosis analyses revealed that there were considerable decreases in percentages of spontaneous apoptosis in young (Y2 group) and elderly (O2 group) people compared with two control groups (both p values <0.001), but no significant difference in children was found. Moreover, percentages of apoptosis with UV-irradiation treatment in three of supplemented groups, significant decreased by 1.4% (p<0.026), 1.9% (p<0.001) and 3.1% (p<0.001) in children, young and elderly people, respectively compared with the placebo groups (Table 4).

Discussion

In this study, we observed a positive association of spontaneous apoptosis of human lymphocytes with age in children, young and elderly people. After 2 months of micronutrient supplementation, there was a significant decrease of intrinsic apoptosis in young and elderly people, as well as a considerable decrease of UV-induced apoptosis in all three age-groups, compared with their controls.

Strengths of our study are a large sample size, a successful randomization, with balanced groups according to characteristics like age, height, weight and body mass index (BMI). Compliance was excellent probably because study subjects were motivated through weekly visits. Moreover, the same samples of fresh blood from each group of children, young and elderly people were collected on the same day, and apoptosis of peripheral blood lymphocytes (PBL) was measured within two hours. We cannot exclude the possibility that cells were damaged or were triggered to undergo cell death. Another study using a lysed whole blood technique found similar results in regards to significantly elevated basal apoptosis in lymphocytes from elderly subjects compared to young controls (22). Therefore, our isolation procedure seems to be rather mild and the detected basal apoptosis may at least partly reflect already existing apoptotic cells.

In our study, the lowest percentage of basal apoptosis was found in children (6.3%). Teaeva, et al reported that there only

Table 2
Characteristics of children, young and old people in supplemented and control groups at baseline

Items	Children			Young people			Old people		
	Mean ± SD	Mean ± SD	p	Mean ± SD	Mean ± SD	p	Mean ± SD	Mean ± SD	p
Subjects									
Groups[1]	C1	C2		Y1	Y2		O1	O2	
No. of subjects	48	40		45	45		50	45	
Completed	45	38		42	42		46	41	
Dropping out	3	2		3	3		4	4	
Characteristics									
Age (years)	10.1±0.7	10.0±0.6	0.905	18.6±0.8	18.6±1.2	0.948	65.3±7.3	65.3±9.0	0.959
Height (cm)	139.2±7.5	137.8±6.4	0.386	169.1±8.4	167.7±8.8	0.394	161.7±8.0	161.6±8.0	0.981
Weight (kg)	33.6±6.5	31.4±4.8	0.264	60.5±10.1	59.5±9.0	0.588	63.6±8.6	61.9±9.9	0.367
Body Mass[2] Index (kg/m^2)	--	--	--	21.1±2.8	21.2±2.0	0.942	24.4±3.2	23.7±3.2	0.234

[1] Six groups: C1, Y1 and O1 were as control groups, and C2, Y2 and O2 as supplemented groups in children, young and old people, respectively; [2] BMI indexes of Children did not be calculated, because the formula is not suitable for children.

Table 3
Changes and differences of plasma micronutrient levels between groups in children, younger and old people after the trial

Indicators	Groups1	Baseline (Mean± SD)	Changes from baseline Mean	95% CI	p value	Differences from control group mean	P value
-carotene (μmol/L)	C1	1.95±0.30	-0.03	-0.23 0.18	0.800	--	--
	C2	1.91±0.33	0.35	0.17, 0.58	0.000	0.38	0.000
	Y1	1.99±0.55	0.10	-0.10, 0.30	0.327	--	--
	Y2	2.07±0.48	0.59	0.39, 0.79	0.000	0.49	0.000
	O1	1.93±0.50	-0.15	-0.35, 0.05	0.145	--	--
	O2	1.83±0.49	0.49	0.24, 0.64	0.000	0.64	0.000
Retinol (μmol/L)	C1	1.83±0.37	-0.10	-0.32, 0.12	0.359	--	--
	C2	1.84±0.26	0.47	0.26, 0.68	0.000	0.57	0.000
	Y1	1.83±0.33	-0.02	-0.23, 0.19	0.850	--	--
	Y2	1.87±0.46	0.84	0.63, 1.05	0.000	0.86	0.000
	O1	1.92±0.25	0.03	-0.18, 0.24	0.773	--	--
	O2	1.86±0.31	0.66	0.46, 0.87	0.000	0.63	0.000
tocopherol (μmol/L)	C1	12.03±2.49	0.09	-1.22, 1.41	0.892	--	--
	C2	12.46±3.01	2.02	0.76, 3.28	0.002	1.93	0.003
	Y1	13.81±3.20	0.61	-0.63, 1.86	0.330	--	--
	Y2	13.58±3.34	4.62	3.38, 5.87	0.000	4.01	0.000
	O1	13.20±1.76	0.03	-0.91, 1.60	0.588	--	--
	O2	12.87±1.92	2.53	1.32, 3.75	0.000	2.19	0.001
Ascorbic acid (μmol/L)	C1	28.9±12.7	5.2	-1.28, 11.62	0.116	--	--
	C2	32.5±12.5	15.5	9.36, 21.73	0.000	10.4	0.000
	Y1	29.6±11.3	-6.0	-12.12, 0.09	0.054	--	--
	Y2	26.2±8.4	17.3	11.15, 23.36	0.000	23.3	0.000
	O1	23.9±16.1	-0.2	-6.39, 5.90	0.938	--	--
	O2	25.2±14.6	16.0	10.01, 21.94	0.000	16.2	0.000
Selenium (μmol/L)	C1	0.95±0.15	-0.03	-0.19, 0.14	0.727	--	--
	C2	0.87±0.15	0.15	-0.00, 0.31	0.056	0.18	0.025
	Y1	0.82±0.44	-0.01	-0.16, 0.15	0.945	--	--
	Y2	0.79±0.44	0.42	0.27, 0.58	0.000	0.43	0.000
	O1	0.86±0.44	-0.01	-0.17, 0.15	0.884	--	--
	O2	0.79±0.43	0.19	0.04, 0.34	0.014	0.20	0.011

[1]Six groups: C1, Y1 and O1 were as control groups, and C2, Y2 and O2 as supplemented groups in children, young and old people, respectively.

was a much lower percentage (2.9%) of apoptosis for full-term newborns (23). However, we found that basal levels of spontaneous apoptosis in freshly isolated PBL are significantly elevated in the groups of elderly and young subjects. This higher susceptibility to apoptosis in aging may be correlated with an increased production and not satisfactory elimination of ROS in aging, thereby leading to apoptosis. Usually, apoptotic cells are eliminated quite fast after generation. A higher percentage of basal apoptotic levels in aged human PBL could indicate an impaired phagocytosis of these cells in the aging population. Several changes in lymphocytes have been observed related to aging: diminished synthesis of growth and survival factors (24-25), impaired intracellular calcium regulation (26), different surface molecular expression (27) and defects of signal transduction (28).

In humans, age affects the in vitro susceptibility to oxidative stress-induced apoptosis (29), and ultraviolet-B radiation results in generation of reactive oxygen species (30). Aging or ultraviolet-B radiation, caused by the accumulation of molecular damage in DNA, proteins and lipids, is also characterized by an increase in intracellular oxidative stress due to the progressive decrease of the intracellular ROS scavenging (31). Our study showed that there was considerable decrease of spontaneous apoptosis in young and elderly people after the trial, which may be attributed to an increase of micronutrient concentrations in plasma and cell contents, and decreasing harmful effects of oxidative stress. Vitamin A, C, and E exhibited protective effects in human lymphocytes by induced ROS generation (32). Similar studies showed that the addition of ascorbic acid, vitamin E, or activated protein C significantly decreased apoptosis rates of endothelial cells, and decreased mortality among those aged 66–69 years by 41% (33-34). Ascorbic acid pre-treated quartz stimulates TNF-alpha release in RAW 264.7 murine macrophages through ROS production and membrane lipid peroxidation (35), and ascorbate protects macrophages against oxLDL-induced oxidant stress and subsequent apoptotic death without impairing their function (36). Oxidative endometrial damage plays an important role in fluoride -induced endometrial toxicity, and the modulation of oxidative stress with vitamin E and ascorbic acid reduces fluoride -induced endometrial damage both at the biochemical and histological level (37). Selenium attenuates ROS-mediated

Table 4
Changes and differences of lymphocyte apoptosis in groups in children, young and old people after the trial

Items	groups	Baseline (Mean± SE)	Changes from baseline			Differences from C group		
			Mean	95% CI	P value	Mean	95% CI	P value
Intrinsic apoptosis (%)	C1	6.6±1.9	0.01	-0.89, 0.91	0.989	--	--	--
	C2	6.1±1.4	-0.65	-1.51, 0.22	0.141	-0.65	-1.53, 0.22	0.142
	Y1	11.4±1.0	0.17	-0.68, 1.02	0.695	--	--	--
	Y2	10.9±1.7	-1.50	-2.35, -0.65	0.001	-1.67	-2.53, -0.80	0.000
	O1	13.8±1.5	0.41	-0.44, 1.27	0.344	--	--	--
	O2	13.8±1.7	-1.89	-2.72, -1.06	0.000	-2.30	-3.15, -1.45	0.000
UV-induced apoptosis (%)	C1	19.3±2.4	-0.90	-2.19, 0.38	0.167	--	--	--
	C2	18.9±2.9	-2.32	-3.55, -1.10	0.000	-1.42	-2.67, -0.17	0.026
	Y1	24.2±1.6	-0.67	-1.88, 0.55	0.281	--	--	--
	Y2	24.6±1.8	-2.58	-3.80, -1.37	0.000	-1.91	-3.15, -0.68	0.002
	O1	33.2±2.3	0.51	-0.71, 1.73	0.413	--	--	--
	O2	32.5±2.7	-2.60	-3.79, -1.42	0.000	-3.11	-4.33, -1.90	0.000

[1]Six groups: C1, Y1 and O1 were as control groups, and C2, Y2 and O2 as supplemented groups in children, young and old people, respectively.

apoptotic cell death of injured spinal cord through prevention of mitochondria dysfunction (38), and effectively inhibits ROS-mediated apoptotic neural precursor cell death in traumatic brain injury (39). These findings provide further confirmation for the notion that the supplementation of diet with antioxidative vitamins and selenium increases the oxidation resistance in human plasma.

Conclusions

In conclusion, we have observed higher levels of intrinsic apoptosis in young and elderly people compared to children which may be related to accumulation of oxidative stress with aging. Moreover, supplementation with moderate amounts of antioxidants may be beneficial to the aging people by decreasing apoptosis.

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