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Low Nourishment of Vitamin C Induces Glutathione Depletion and Oxidative Stress in Healthy Young Adults

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ABSTRACT: The present study was conducted to assess the status of vitamin C among healthy young adults in relation to serum antioxidant parameters [glutathione (GSH), thiols, and total antioxidant capacity, (TAC)], and oxidative stress markers [malondialdehyde (MDA), and nitrites plus nitrates (NN)]. A prospective study included 200 young adults, and their dietary intake was assessed by using food diaries. Fasting plasma vitamin C, serum levels of GSH, thiols, TAC, MDA, and NN were measured using biochemical assays. It was observed that 38% of the enrolled subjects, n=76, had an adequate dietary intake of vitamin C (ADI group). Meanwhile, 62%, n=124, had a low dietary intake of vitamin C (LDI group) as compared to the recommended dietary allowances. The fasting plasma level of vitamin C was significantly higher in the ADI group as compared to the LDI group. Oxidative stress in the sera of the LDI group was evidenced by depletion of GSH, low thiols levels, impairment of TAC, an elevation of MDA, and increased NN. In the ADI group, positive correlations were found between plasma vitamin C and serum antioxidant parameters (GSH, thiols, and TAC). Meanwhile, the plasma vitamin C was negatively correlated with serum MDA and NN levels. This study reveals a significant increase of oxidative stress status and reduced antioxidant capacity in sera from healthy young adults with low intake of the dietary antioxidant, vitamin C.

Keywords: vitamin C, oxidative stress, young adults, non-communicable diseases

INTRODUCTION

During adulthood, there is increased evidence for the participation of free radicals in the etiology of non-communicable diseases, including cancer, type 2 diabetes, and cardiovascular diseases (1). Free radicals induce oxidative reactions like initiation of the peroxidation of the cell membrane lipids bilayer leading to the accumulation of lipid peroxides, direct inhibition of mitochondrial respiratory chain enzymes, fragmentation or random cross linking of molecules like DNA, enzymes and proteins which ultimately leads to cell death (2). Vitamin C, a dietary antioxidant, neutralizes the free radicals, protects the cellular membranes against oxidative damage, and prevents damage to proteins, enzymes, and DNA (3). A wide range of studies has indicated that vitamin C supplementation delays the incidence of non-communicable diseases and improves human health and wellbeing (4).

Glutatione (GSH, γ-glutamylcysteinylglycine), an intracellular antioxidant, provides cells with its reducing milieu which is essential for maintenance of the thiols of cellular proteins, of cellular vitamin C, and α-tocopherol in the cell membrane (5). Cells contain predominantly the reduced form of GSH, which interacts with endogenous or exogenous oxidative stress insults with the formation of oxidized glutathione, requiring a nicotinamide adenine dinucleotide phosphate-oxidase-dependent pathway for its reduction to GSH (6). Through its reaction, GSH protects cells against oxidative damage, and it has been documented that cellular GSH depletion is involved in the pathogenesis of oxidative stress-mediated non-communicable diseases (7). GSH depletion increased superoxide anions formation in experimental animals challenged with various oxidative stress insults (8).

Diets rich in fresh fruits and vegetables are protective against non-communicable and degenerative diseases. The beneficial effects of fruits and vegetables are hypothesised to be mainly due to vitamin C and other phytochemicals (9,10). This has been recognised in the recent increase in the US daily reference intake of vitamin C for women and men (75 and 90 mg/day, respectively),

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with an additional 35 mg/day recommended for smokers (11,12). However there is a lack of research reporting the effect of vitamin C as a dietary modulator of GSH; therefore, we conducted this prospective cross-sectional study to evaluate the status of vitamin C in relation to oxidative stress markers among healthy Omani young adults.

SUBJECTS AND METHODS

Study subject and setting

A prospective, 6 months, cross-sectional study was conducted at Sultan Qaboos University. The study included two hundred young adults, newly admitted male university students. All study subjects were recruited on voluntary basis. They were healthy, non-smokers, and free of endocrine disorders, eating disorders, gastrointestinal diseases, or any non-communicable diseases (cardiovascular diseases, diabetes, and hypertension). None of the study participants were consuming any vitamin C supplementation or any multivitamins. The study was approved by the Research Ethics Committee of the Ministry of Health, Sultanate of Oman (Ref. MH/385).

Study protocol and dietary assessment

On day 1 of the study, all study subjects were informed about the study questionnaire, and were encouraged not to alter their routine dietary habits. The prospective dietary intake was self-estimated by the study subjects using food diaries, and they were asked to report the portion size for each food item consumed during a period of 6-months starting from day 1 of the study recruitment until day 180, the last day of the study. There was a frequent follow up phone calls with all subjects to assure their compliance with the study protocol, all phone calls were conducted on week 1, week 2, week 4 (1 month), week 6, week 8 (2 months), week 10, week 12 (month 3), and week 24 (6 months, last day of the study).

All study subjects recorded details of foods and beverages consumed. All study subjects provided portion sizes, brand names, cooking, and preparation methods. The Food Processor software version 10.2 (Esha Research Inc., Salem, OR, USA) was used to calculate the average daily nutrient intakes of total energy intake, protein, fat, carbohydrates, and dietary antioxidants (vitamin C, β-carotenes, selenium, and vitamin E) as estimated from the number of servings consumed, portion sizes and nutrients content for all the reported foods by each study subject.

Anthropometric measurements and physical activity assessment

Weight (kilograms) was measured by TANITA scale (Model No. TBF-410 AS, Tanita Corporation, Tokyo, Japan). The Stadiometer was used to measure the standing height to the nearest 0.5 cm; height measurement was done with the subject standing without shoes, with the back against the wall tape and the visual axis horizontal. Body mass index (BMI) for every subject was calculated as weight in kilograms divided by height in meters squared (kg/m^2) and was categorized as normal, 18.50～24.99, overweight, 25.00～29.99, and obese, ≥30.00 (13).

The intensity of all physical activities performed by the study subjects was calculated as the sum of each type of activity weighted by its energy equivalent and expressed as the metabolic equivalent of task per week (METs-min/week) as follows:

$$\text{METs-min/week} = \sum(\text{energy equivalent of the task} \times \text{number of days} \times \text{duration per min})$$

The energy equivalent is 3.3 for walking, 4 for moderate activity and 8 for vigorous activity. The recommended 60 min per day of moderate activity for young adults is equivalent to 1680 METs-min/week and calculated as $\sum$(60 min×4 METs×7 d), and physically active subjects are those who accumulated a score≥1680 METs-min/week while and physically inactive are those who accumulated<1680 METs-min/week (14).

Blood collection

On the last day of the trial, day 180, all study subjects were instructed to fast overnight, and from each study subject; 5～10 mL of venous blood was collected through a peripheral venous catheter into both heparinazed and plain top vacutainer tubes. When the blood reached room temperature, the samples were centrifuged at 6,000 rpm for 10 min at 4°C, and aliquots of plasma and serum were transferred to 1.5 mL Eppendorf tubes and stored at −80°C for subsequent biochemical analyses. For analysis of vitamin C, 500 μL plasma was immediately transferred to Eppendorf tubes and mixed with an equal volume of 10% metaphosphoric acid and dithiothreitol before the samples were frozen. The dithiothreitol reduces any dehydroascorbic acid present in the sample to ascorbic acid permitting the measurement of total vitamin C in plasma specimens.

Biochemical analyses

Determination of plasma Vitamin C: Analyses of vitamin C were performed using an HPLC. The samples (diluted 1:1 with 10% m-phosphorous acid) were thawed at 4°C and centrifuged at 10,000 *g* for 2 min. Twenty μL aliquots of the clear supernatants were injected. Vitamin C was determined with a spectrophotometric detector at the wavelength of 262 nm. The HPLC pump was a

880-PU and the detector a 875-UV, both from Jasco (Japan Spectroscopic Company Ltd., Tokyo, Japan), and the injector was a Rheodyne 7125 from Rheodyne, Inc., (Cotati, CA, USA). The analytical column was a Chromolith® Performance column (100×4.6 mm, RP-18e, Merck KGaA, Gernsheim, Germany). The mobile phase consisted of 200 mmol/L KH_2PO_4, pH 3.0 and the flow rate was 2.5 mL/min. Standard curves with a concentration of 0～100 μmol/L vitamin C were prepared and used for the quantification. Stock solutions of vitamin C with a concentration of 10 mmol/L were first prepared in water and then diluted further with 10% metaphosphoric acid.

Measurements of serum oxidative stress markers: Serum oxidative stress markers were measured according to the manufacturer's instructions (BioVision, Inc., Milpitas, CA, USA) in each kit: GSH by assay kit K251, the total antioxidant capacity (TAC) by assay kit K274, malondialdehyde (MDA) by assay kit K739, and nitrites and nitrates (NN) by assay kit K262.

Thiols were determined with Ellman's reagent (15). Briefly, 50 μL of plasma were mixed with 1.0 mL of 0.1 M Tris, and 10 mM EDTA at pH 8.2. The absorbance was determined at 412 nm, and 40 μL of 10 Mm Ellman's reagent in methanol were added to the sample. The absorbance obtained before the addition of Ellman's reagent was subtracted from that obtained after incubation with Ellman's reagent. A control containing Ellman's reagent only was included, and the concentration of thiol groups was calculated using a molar extinction coefficient of 13,600 $M^{-1} \cdot cm^{-1}$ at 412 nm.

Data and statistical analysis

The collected data were expressed as mean±standard deviation (SD) for the continuous variables or as percentages for categorical variables. The GraphPad Prism (version 5.0, Graphpad Software Inc., La Jolla, CA, USA) was used to compute the unpaired Student's *t*-test, and simple correlation coefficients (r) were quantified for assessing the correlations between vitamin C status and biochemical indices. $P<0.05$ is considered statistically significant.

RESULTS

General characteristics

A cross sectional survey was conducted in March 2014 to study the vitamin C status and biochemical determinants of oxidative stress among 200 male young adults attending Sultan Qaboos University in Muscat governorate based on a self-administered questionnaire. The majority of study subjects (n=180; 90%) reported a monthly family income enough to cover their expenses either with savings, while a small proportion reported a monthly family income without savings (n=20, 10%). Chronic health problems calling for medical care were not reported by any study subjects. The mean age of the study subjects was comparable with no statistical significant differences, $t=1.21$, $P>0.05$, 22.4±1.2 years for the adequate dietary intake of vitamin C (ADI) group and 22.6±1.1 years for the low dietary intake of vitamin C (LDI) group. The BMI measurements of the ADI and the LDI groups revealed normal BMI with no significance differences (19.7±0.4 and 19.2±0.1 kg/m^2, respectively), $P>0.05$.

Types of exercises more frequently reported by study subjects were walking (n=90, 45%), running (n=70, 35%), and swimming (n=40, 20%). All study subjects met the recommended level of physical activity equivalent to ≥1680 METs-min/week. The use of metabolic equivalents to express the levels of physical activity allowed the identification of young adults who meet the recommended levels with certain degree of objectivity.

The study subjects were categorized based on the adequacy of their dietary vitamin C intake, as compared to the estimated average requirements (EAR) for vitamin C (75 mg/d) for males aged 19～30 years (16). ADI group was 38% of the enrolled subjects (n=76) with an adequate daily intake of vitamin C>75 mg/d. LDI group was 62 % of the enrolled subjects (n=124) with a low daily intake of vitamin C<75 mg/d.

Dietary intake of vitamin C and dietary antioxidants nutrients

All study subjects did not have any change in their dietary pattern than the usual routine during the study period. The daily dietary intake for macronutrients and vitamin C for the ADI and LDI groups is presented in Table 1. It was observed that both groups consumed similar amounts of carbohydrate, protein, fat, and total energy intake ($t=0.374$, $t=1.086$, $t=0.469$, and $t=0.419$, respectively, $P>0.05$). Meanwhile, the ADI group had a significant higher intake of vitamin C compared to the LDI group ($t=38.78$, $P<0.05$). Data revealed that all subjects (ADI and LDI groups) had normal daily intake of antioxidant nutrients (vitamin E and selenium) compared to the EAR (16). They also had a comparable level of daily intake of β-carotenes, $P>0.05$.

Serum oxidative stress status

As presented in Table 2, significant lower levels of serum antioxidant parameters; GSH, thiols, and TAC (8.21±0.89 μmol/L, 385.6±42.3 μmol/L, and 28.92±1.1 mmol/L, respectively) were observed among the LDI group as compared to the ADI group (28.92±1.1 μmol/L, 456.2±38.1 μmol/L, and 122.3±12.89 mmol/L, respectively), $P<0.05$. By contrast, oxidative stress indices (MDA and NN) were significantly increased in the LDI

Table 1. Distribution of the young adults according to daily intake of macronutrients total energy, and dietary antioxidants nutrients

Nutrients	ADI group[1)]	LDI group[2)]	Test of significance
Carbohydrates (g/d)	386.6±22.2	385.4±21.9	t=0.374, P=0.709
Protein (g/d)	64.6±6.7	63.5±7.1	t=1.086, P=0.279
Total fat (g/d)	96.2±10.9	95.5±9.8	t=0.469, P=0.639
Total energy intake (kcal/d)	2,670.6±312.8	2,655.1±210.6	t=0.419, P=0.676
Vitamin C (mg/d)	86.5±7.5	48.2±6.3*[3)]	t=0.469, P=0.001
β-Carotenes (mg/d)	2.19±0.85	2.31±0.65	t=1.125, P=0.262
Selenium intake (μg/d)	70.81±13.02	69.82±15.01	t=0.476, P=0.635
Vitamin E (mg/d)	17.09±2.31	17.61±4.69	t=0.901, P=0.369

Values are expressed as mean±SD.
[1)]ADI group: Adequate dietary intake of vitamin C (n=76).
[2)]LDI group: Low dietary intake of vitamin C (n=124).
[3)]The asterisk denotes that data are significantly lower than the ADI group, $P<0.05$. The estimated average requirements (EAR) for vitamin C, selenium, and vitamin E (75 mg/day, 55 μg/day, and 15 mg/day, respectively).

Table 2. Distribution of the young adults according to serum levels of antioxidant parameters and oxidative stress markers

Oxidative stress indices[1)]	ADI group[2)]	LDI group[3)]	Test of significance
GSH (μmol/L)	28.92±1.1	8.21±0.89*[4)]	t=145.8, P=0.001
TAC (mmol/L)	122.3±12.89	69.6±11.2*	t=30.48, P=0.001
Thiols (μmol/L)	456.2±38.1	385.6±42.3*	t=11.89, P=0.001
MDA (μmol/L)	1.43±0.14	3.19±0.2#	t=67.25, P=0.001
NN (μmol/L)	22.11±4.1	80.23±16.8#	t=29.60, P=0.001

Values are expressed as mean±SD.
[1)]GSH, glutathione; TAC, total antioxidant capacity; MDA, malondialdehyde; NN, nitrites plus nitrates.
[2)]ADI group: Adequate dietary intake of vitamin C (n=76).
[3)]LDI group: Low dietary intake of vitamin C (n=124).
[4)]The asterisk denotes that data are significantly lower than controls group and the hash mark denotes that data are significantly higher than controls group, $P<0.05$.

Table 3. Correlation between plasma vitamin C level and biochemical characteristics in the sera of ADI and LDI groups

Measurements[1)]	ADI group		LDI group		Strength of correlation
	r	P	r	P	
Dietary vitamin C	0.865	0.001	0.971	0.015	Strong positive correlation
Serum MDA	−0.69	0.002	−0.71	0.005	Intermediate negative correlation
Serum NN	−0.59	0.003	−0.61	0.003	Intermediate negative correlation
Serum GSH	0.43	0.03	0.54	0.02	Intermediate positive correlation
Serum thiols	0.34	0.012	0.46	0.004	Intermediate positive correlation
Serum TAC	0.36	0.02	0.58	0.013	Intermediate positive correlation

[1)]MDA, malondialdehyde; NN, nitrites plus nitrates; GSH, glutathione; TAC, total antioxidant capacity.
If value of (r) was positive this indicated a direct relationship, i.e., when a plasma vitamin C increases, the biochemical parameter increased. If value of (r) was negative this indicated an inverse relationship between the two variables, i.e., when a plasma vitamin C increases, the biochemical parameter decreased.
All data were significantly correlated ($P<0.05$).

group compared to the ADI group, $P<0.05$, Table 2.

Plasma level of vitamin C

It was noted that the ADI group had higher levels of plasma vitamin C levels compared to the LDI group (56.78±9.4 and 22.92±4.7 mol/L, respectively). The differences were significant (t=33.83, $P<0.05$). As summarized in Table 3, it was observed that there was a strong positive correlation between daily intake and plasma levels of vitamin C in both the ADI and LDI groups. No correlation was observed between plasma vitamin C, BMI, and age. Meanwhile in both ADI and LDI groups; the plasma vitamin C was negatively correlated with serum MDA and NN levels, and there was an intermediate positive correlation between plasma vitamin C and serum levels of GSH, thiols and TAC.

DISCUSSION

The steady increase of non-communicable diseases in Oman is mainly attributed to the changes in the nutritional habits of the Omani population (17). Health promotion activities are undertaken all over the globe to in-

crease the dietary intake of dietary antioxidants, including vitamin C, during adulthood stage of life (18). Epidemiological studies have found significant associations of higher intake of vitamin C with reduced risk of oxidative stress mediated non-communicable diseases such as cancer, cardiovascular diseases, diabetes, and hypertension (19). Fruits and vegetables are rich in dietary antioxidants, vitamin C, β-carotenoids, and selenium. High consumption of foods rich in these antioxidants results in a decreased risk for such non-communicable diseases. The present study has indicated that the mean daily dietary intake for vitamin C among healthy young adults was positively associated with an increase in plasma vitamin C level and serum antioxidants parameters, including GSH, total antioxidant capacity, and thiols. On the contrary, low intake of dietary vitamin C was negatively associated with serum oxidative stress parameters (MDA and NN).

GSH is the intracellular non-protein thiol in all mammalian cells, and it plays an important biological role in detoxification functions (20). Thiols contribute to the extracellular antioxidant capacity and are essential for the protection against different oxidative insults (21). Our data suggested that depletion of GSH, lower thiols levels, and impairment of TAC were common in the sera of the LDI group indicating a cellular oxidative stress condition. In addition, there was as an increase in reactive oxygen species products, MDA and NN, leading to an imbalance between dietary antioxidants and oxidative stress insults.

Oxidative stress decreases tissue GSH levels and decreases cellular redox status; therefore, restoring GSH levels by dietary approaches might be an effective preventive measure against various oxidative stress insults (reactive oxygen species and xenobiotics). Recent research proposed different therapeutic approaches for the reversal of GSH depletion by administration of GSH esters, which are readily transported into cells and hydrolysed to form intracellular GSH (22), or by cysteine prodrug supplementation that is required as a precursor for the *de-novo* synthesis of GSH (23).

In this context, it might be speculated that adequate intake of vitamin C acts as a primary prevention intervention for non-communicable diseases among young adults. A number of studies have reported that the plasma vitamin C concentrations are higher in young adults supplemented with vitamin C (24,25). In the National Health and Nutrition Examination Surveys, it was observed that food diversity was a major determinant for vitamin C status (26).

This current study presents new data, using a novel approach, that the increase in serum MDA and NN levels in association with the decrease of serum antioxidant parameters (GSH, thiols, and TAC), and low intake of vitamin C comforts the hypothesis of an oxidative damage in the LDI group, the long term or chronic trigger for non-communicable diseases incidence during adulthood stage of life. The monitoring of serum markers of the oxidative stress status could prevent the development of non-communicable diseases. Our observation that plasma vitamin C directly correlates with serum antioxidant parameters (GSH, thiols, and TAC), opens new avenues in the monitoring and also the primary prevention of non-communicable diseases.

CONCLUSION

In conclusion, there was a significant increase of oxidative stress status and reduced antioxidant capacity in sera from healthy young adults with low dietary intake of vitamin C. However our data do not allow to discriminate whether the observed changes in the redox status are causes or consequences of low vitamin C status.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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