Design for a study to determine optimal dosage of ascorbic acid and alpha-tocopherol in humans

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Abstract Some clinical trials of vitamins C and E have neglected important design features. Our objective was to demonstrate a detailed design that includes essential elements for an effective study of these vitamins *in vivo*. While taking 400 IU (international units) of vitamin E, subjects took different dosages of vitamin C during three distinct periods. Dosages were 200 mg in food, 500 mg as supplements twice a day (500×2), and 1,000 mg as supplements twice a day (1000×2). Ten participants spent 3 weeks at each dosage before plasma was drawn on two consecutive days. Final samples were taken after a week with no supplementation. Selected by investigators at four institutions, endpoints were protein carbonyls, TBARs (thiobarbituric reactive substances), and Heinz body formation in RBCs (red blood cells). TBARs and protein carbonyls did not change significantly with dosage. However, Heinz body formation increased at the highest and lowest intakes of vitamin C. Even with daily vitamin E,

Heinz bodies were significantly fewer at 500×2 . Results indicate that even with 400 IU vitamin E daily, it is possible to distinguish the effect of different levels of vitamin C with Heinz bodies. This effect may be due to pro-oxidant action of vitamin C or to prolonged survival of RBCs.

Key words antioxidants · ascorbic acid · free radicals · Heinz bodies · protein carbonyls · TBARs · tocopherol · vitamin C · vitamin E

Abbreviations

AA	ascorbic acid/vitamin C
AGE	American Aging Association
ANOVA	Analysis of variance
AT	dl-alpha-tocopheryl-acetate/vitamin E
DCIP	dichlorophenol-indophenol
DNP	dinitrophenol
ELISA	enzyme-linked immunosorbent assay
IU	international units
NIH	National Institutes of Health
RBC	red blood cells
SUP	supplement
TBARS	thiobarbituric acid reactive substances
UL	Upper limit
500×2	500 mg AA twice a day
1000×2	1000 mg AA twice a day

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Introduction

Since the original proposal of the free radical theory of aging by American Association of

Aging (AGE) founder Dr. Denham Harman, free radicals have been shown to be involved in many age-associated diseases (Haan 2003; Beckman and Ames 1998). Extensive research has indicated that free radicals contribute to diseases such as cancer, diabetes mellitus, Parkinson's disease, senile cataracts, and arteriosclerosis (Haan 2003; Beckman and Ames 1998). Antioxidants vitamin C (AA, water-soluble) and vitamin E (AT, fat-soluble) trap free radicals and thus may be protective against age-associated diseases. There is considerable epidemiological evidence that doses of AA and AT above the recommended daily value are beneficial (Losonczy et al. 1996), protecting the nervous system, immune system, vision, and circulatory system from free radicals (Packer et al. 2002). However, studies such as those that recently examined antioxidants AA and AT (Huang et al. 2002; Bruunsgaard et al. 2003; Jacob et al. 2003; Fang et al. 2002) often overlook important design features. These include appropriate dosages, sampling times, and endpoints. The objective of this study was to design and test in humans a protocol to determine a useful vitamin C and E dosage to minimize detectable *in vivo* oxidative damage by free radicals.

Identifying the mechanisms by which AA and AT can reduce plaque in arteries, maintain arterial compliance, and provide other medical benefits (Heitzer et al. 1996) helps explain their beneficial functions. AGE held a Consensus Conference in Seattle in 1999 (Meydani 1999) to consider optimal doses of AA and AT (Ordman 1999). It was decided that AT in the range of 200–400 IU and AA in the range of 200–1,000 mg daily warranted consideration. A reservation about high doses of AA is that oxalate excretion might contribute to kidney stones (Levine et al. 1996), but no peer-reviewed evidence to support the kidney stone hypothesis has been found (Curhan et al. 1996).

Because most of a high dose of AA is excreted over 12 h (King et al. 1994), it is important to control the time between tablet consumption and *in vivo* sampling. The first measurable endpoint used to determine a pharmacological dosage for AA was urinary excretion (King et al. 1994), which demonstrated that 500 mg of oral AA is

absorbed over 4 h and excreted over the next 12 h. It has been found that large oral doses of other water-soluble nutrients, such as calcium (Cone et al. 1996), are also excreted over 12 h.

While fat-soluble vitamins are not required every 12 h, evidence is accumulating that dosages of AT substantially above currently recommended daily values may be beneficial. Brown et al. demonstrated that 560 mg AT are more effective in lowering the level of erythrocyte peroxidation than 140 or 1,050 mg (Brown et al. 1997). Meydani et al. demonstrated that 200 mg AT are more effective than 60 or 800 mg in obtaining effective immune response to vaccination (Meydani et al. 1997). Such studies have established that doses above the recommended daily value are useful for measurable health benefits. The goal of this study was to identify dosages and endpoints that will be useful for a larger study of the combination of AA and AT.

Materials and methods

Study population

Beloit College Institutional Review Board approval was received before experimentation began. Ten healthy participants (eight female, two male) from Beloit College volunteered for the study. The median group characteristics were age 23 years, height 178 cm, and weight 66 kg. Only one participant smoked, approximately five filter cigarettes per day.

Regimen and compliance

The subjects stopped taking any supplements for a week prior to participating. None were on medication. Vitamin regimens and timeline are shown in Tables 1 and 2 respectively. Subjects were assigned to three consecutive regimens, each lasting three weeks with a one-week wash out period between regimens. Regimens were randomized in case of order effects. All participants were instructed about the AA content of foods commonly available to them. A list of foods in the dining hall and AA-containing drinks was provided to participants. By individual discussion

Table 1 Dietary regimens.

Regimen	Morning		Evening	Diet	Total
	AT, iu (Sup)	AA, mg (Sup)	AA, mg (Sup)		
1	400	None	None	Trained to eat 200 mg of AA from food	200 mg AA as food
2	400	500	500	Avoided AA-enriched drinks	500 × 2
3	400	1,000	1,000	Avoided AA-enriched drinks	1000 × 2
Final	None	None	None	Avoided AA-enriched drinks	Minimal

Subjects spent 3 weeks on each regimen in randomly assigned order. Each completed all three regimens. The final regimen provided internal controls for low vitamin C intake.

Abbreviations: AA, ascorbic acid/vitamin C; AT, dl-alpha-tocopheryl-acetate/vitamin E; Sup, supplement; 500 × 2, 500 mg AA twice a day; 1000 × 2, 1000 mg AA twice a day.

with the project supervisor, each participant selected what they would eat that would provide the 200 mg AA during the food-based regimen. They maintained a food and supplement record for the 3 days prior to blood sampling and during the day of sampling, at which time the records were submitted. Signed questionnaire forms, food record, and frequent emails and phone calls indicated that all participants complied with every regimen. At the start of each 3-week regimen, compliance forms, food diaries, regimen instruction, and the correct number of tablets (Nature's Made 500 mg AA and 400 IU AT) were provided.

Sampling

On days 20 and 21 of each 3-week period, blood samples were drawn by finger prick from each participant 4–12 h after morning vitamins were taken, and dietary records were collected. Participants were asked to resume their personal dietary patterns without supplements for 6 days

between regimens. This is sufficient to restore AA to unsupplemented levels (King et al. 1994). As AT supplementation was invariable among regimens, baseline levels were not determined. After the last regimen, participants discontinued all antioxidant supplementation for more than 7 days so AA levels could decrease. A final blood sample was then taken to provide baseline values. Serum for TBARs and protein carbonyls was stored at -70°C until analysis. Heinz body levels were determined immediately after blood collection.

Study endpoints and laboratory methods

TBARs (a crude index of lipid peroxide levels) were determined by the Yagi method (Yagi 1998) at the University of Arizona. In this method, serum samples are treated to remove water-soluble substances. Then thiobarbituric acid is added to the serum samples and reacts with lipid peroxides, producing a measurable fluorescent end product.

Table 2 Timeline.

Regimen	1			2			3			Final	
Time (days)	7	19	2	7	19	2	7	19	2	6	2
Action	A	B	C	A	B	C	A	B	C	D	E

A, Avoided vitamin C-enriched foods and all supplements; B, randomly assigned to regimen 1 (200 mg as food), 2 (500 × 2), or 3 (1000 × 2) for 3 wk. Frequent regimen reminders were sent by e-mail and telephone; C, blood drawn by finger prick between 4 h and 12 h after morning supplements; D, vitamin C- and E-rich foods and all supplements were avoided; E, two blood samples were taken 24 h apart.

Dietary logs were maintained for four consecutive days including blood sampling.

Protein carbonyls were assayed with the Zentech PC Test (Winterbourn and Buss 2002) at the Linus Pauling Institute. In this method, protein is treated with dinitrophenol and then absorbed onto an enzyme-linked immunosorbent assay (ELISA) plate. The plates are washed and stained with an enzyme-linked antibody specific to the DNP-protein complex. The amount of carbonyl is determined by enzyme assay of the plate-bound complex.

For Heinz body counting (Beutler et al. 1995), stained plasma smears were labeled in code and counted in a blinded fashion by three individuals and data were compiled and analyzed.

Statistics

Mean and standard deviations were found for TBARS and protein carbonyls. As assumptions for parametric analysis were met for the Heinz body data, an analysis of variance (ANOVA) between groups was performed using the statistical software JMP to determine significant differences between treatments, the limit of significance being set at $p = 0.05$.

Results

Table 3 shows that neither TBARS nor protein carbonyl levels were significantly different for any of the three regimens, or following a week of vitamin depletion. Free radical damage indicated by the biomarkers of TBARS or protein carbonyls was the same throughout the study. These serum-based peroxidation endpoints, TBARS and protein carbonyls, indicate that people retain sufficient AT to prevent elevated peroxidation.

In order to test the assumptions for an ANOVA of the Heinz body data, both a Shapiro–Wilkes test for normality and a Bartlett’s test for equal variances were performed. The data were normally distributed among every diet group. As the assumptions were met, an ANOVA was performed, resulting in a significant difference among diet groups ($p = 0.002$). Subsequently, a Dunnett’s test was performed to ascertain the differences between the experimental groups and the control. The 500×2 and 1000×2 were significantly different from the control. There were significantly fewer Heinz bodies at 500×2 .

Heinz bodies differed significantly with AA dosage. Table 4 shows the absolute number of Heinz bodies found in participants taking different levels of AA. The highest level of peroxidative damage in hemoglobin was indicated by Heinz bodies in the group avoiding AT and AA. There was no statistically significant difference in Heinz bodies between 1000×2 AA and 500×2 AA. However, both 500×2 and 1000×2 regimens were significantly different ($p < 0.002$) from 200 mg AA in the diet.

Discussion

A primary goal of this paper was to discuss an effective design for a study to determine an optimal human dosage of the antioxidant AA. Four criteria should be regarded as important in the design of effective study of antioxidants: (1) Selection of vitamin dosages should be justified based on prior studies with useful endpoint data. (2) Time from intake of AA supplements to

Table 3 TBARS and protein carbonyls as a function of AA intake.

Regimen	AA intake	TBARS			Protein carbonyls		
		<i>n</i>	Mean nmol/ml serum	SD	<i>n</i>	Mean nmol/mg protein	SD
1	200 mg as food	19	3.63	0.59	21	0.007	0.008
2	500×2	13	3.74	0.44	18	0.008	0.007
3	1000×2	13	3.82	0.80	20	0.007	0.009
Final	Minimal	9	3.50	0.39	7	0.005	0.012

Abbreviations: AA, ascorbic acid/vitamin C; 500×2 , 500 mg AA twice a day; 1000×2 , 1000 mg AA twice a day.

Table 4 Heinz bodies as a function of AA intake.

Regimen	AA intake	Mean ^a	SEM
1	200 mg as food	2.44	0.14
2	500 × 2	1.73*	0.22
3	1000 × 2	2.12*	0.15
Final	Minimal	2.77	0.15

^a Values are means for Heinz bodies found in all blood samples for all individuals during periods 3 and 4.

* $p < 0.002$.

Abbreviations: AA, ascorbic acid/vitamin C; 500 × 2, 500 mg AA twice a day; 1000 × 2, 1000 mg AA twice a day.

sampling must be in the range of 4–12 h to ensure AA has been absorbed and not eliminated. (3) A variety of endpoints such as urinary excretion, white blood cell saturation, plasma concentration, TBARs, protein carbonyls, Heinz bodies, arterial compliance, and signaling processes at the cellular level (Carcamo et al. 2004) may measure different effects. (4) Oxidative endpoints may be influenced by synergistic or independent effects of antioxidants. These criteria were considered in the design of the current study and are discussed individually below.

1. This study examined different dosages of water-soluble AA in the presence of fat-soluble AT. For AT dosage, a consensus conference convened by AGE considered dosages between 200 and 400 IU optimal. In this study 400 IU AT was selected because several clinical studies have used that dose in their trials (Brockes et al. 2003; Winklhofer-Roob et al. 1995; Steinberg and Chait 1998). The concentration of RBC AT is then approximately 30 $\mu\text{mol/g}$ Hb (Brown et al. 1997). Three dosages of AA above the recommended daily value were evaluated based on the findings of three key previous studies (Levine et al. 1996; King et al. 1994).

a) Dosage evaluation from the endpoint of urinary excretion according to Curhan et al. (King et al. 1994). In that study, subjects consumed various dosages of AA supplements, and their urine was tested for excreted AA using a dichlorophenol-indophenol (DCIP) assay. It was found that to achieve a detectable level of AA in the urine of all participants, 500 mg must be consumed. The detectable level

lasted for only 12 h. This was consistent with a dosage of 500 mg every 12 h for a person to maintain elevated AA levels.

b) The second dosage evaluated was 200 mg of AA per day obtained solely from the diet. This dosage is based on a study conducted at the National Institutes of Health (Levine et al. 1996). After an original urinary excretion study was published in AGE, the NIH trial was conducted with a similar design, finding that the 500 mg twice-a-day dosage produced a serum concentration of 71.5 μM AA. The NIH study confirmed the saturation levels of the original study, but suggested a different endpoint for selecting an optimal dosage. The alternative endpoint suggested was the saturation with AA of particular blood cell populations: neutrophils, monocytes, and lymphocytes. The 200 mg level could be achieved without supplements if food containing high levels of AA are consumed. A second dosage recommendation of 200 mg per day through food was thus established, which provides a serum concentration of approximately 65.8 μM .

c) The third dosage in this study was 1,000 mg twice a day, giving a serum concentration of approximately 76.9 μM , which is the UL (upper limit) recommended by the Food and Nutrition Board in the latest federal guidelines for nutrition. This serum concentration of AA is not significantly different from that obtained at a dosage of 500 × 2 (Levine et al. 1996).

2. Timing of dosing and sampling is essential to a meaningful study of antioxidant action. The

two major studies (Levine et al. 1996; King et al. 1994) comparing effective dosages of AA recognized that AA is water-soluble and must be taken twice daily to provide stable concentrations. Studies will be flawed if AA is taken once a day and sampling time for subjects is not carefully controlled.

3. Given the ongoing discussion of free radicals and antioxidants (De Zwart et al. 1999; AGE 2003; Annual Meeting of the Linus Pauling Institute 2003; NIH 2003), methods should be considered that are useful in the range of antioxidants being evaluated. Discussions with colleagues demonstrated the controversy over appropriate endpoints to measure free radical damage *in vivo* (Mayne 2003). Johnston and Cox (Johnston and Cox 2001) have shown that Heinz body analysis is useful at high dosages of AA. Heinz bodies are denatured hemoglobin clumps caused by oxidation that are found *in vivo* in RBCs (Lewis et al. 1995). Because of the resources and samples available for this study, endpoints selected included TBARS (Yagi 1998), protein carbonyls (Winterbourn and Buss 2002), and Heinz body formation (Beutler et al. 1995).
4. Because AA and AT may interact *in vivo*, this trial was designed to control intake of both AA and AT. For instance, low-density lipoprotein (LDL) is subject to free radical oxidation, and may be protected by AT present in LDL. However, AA present in plasma may be able to regenerate AT in LDL. Biomarkers are likely to be sensitive to the level and effect of both antioxidants.

This trial demonstrates that AA and AT may have utility in reducing free radical damage when consumed at levels above the recommended daily value. As shown in Table 3, TBARS and protein carbonyl biomarkers showed no significant elevation in damage even when AA intake was less than 60 mg/day. It is also possible that without an AT supplement, a longer time (greater than 10 weeks) on AA supplements would be necessary to see any effect on oxidative stress markers (Carty et al. 2000). The AT combined with minimal AA was adequate to prevent measurable peroxidation. As AT is fat-soluble, it remained in

the participants at a level that was still protective even after a week without supplementation (Burton and Darosewska 1996; Traber 1999; Burton et al. 1990). This 7-day washout period was selected because it was sufficient to reduce AA levels and was acceptable to the IRB.

Table 4 shows that Heinz body analysis may be useful to assess the benefits of high dosages of antioxidants (Johnston and Cox 2001). Heinz body results are consistent with the hypothesis that 500×2 is significantly more effective in reducing hemoglobin damage. Data are consistent with a disadvantage to consumption of 200 mg or $1,000 \times 2$ per day. While the effect of 500×2 and 1000×2 on blood concentration of AA is not statistically significant (Levine et al. 1996), there is a significant difference in the formation of Heinz bodies between 200 mg from food and 500×2 . Pro-oxidative effects have been observed in dosage comparison studies of AT (Brown et al. 1997; Meydani et al. 1997). The data of Table 4 indicates that pro-oxidative effects may also be found at higher dosages of AA.

Our results are consistent with the latest evaluation of the Food and Nutrition Board. The recommended daily value of AA was raised significantly and a UL of 2,000 mg was established (Hathcock et al. 2005). A UL is justified because it has been shown *in vitro* and suggested in theory that AA may cause pro-oxidant action *in vivo* (Poulsen et al. 1998). Table 4 indicates further study is warranted using Heinz body analysis for possible pro-oxidant effects above the 500×2 dosage. However, the RBCs were not fractionated to distinguish old from young cells. Heinz bodies could influence the lifespan of the RBCs (Jakubowska-Solarska and Solski 2000). Future studies should consider fractionating RBCs before performing Heinz body analysis to eliminate the concern that RBC lifespan is altered by antioxidant supplementation.

Consumption of AA at a level of 200 mg to 500×2 and AT at 200–400 mg was evaluated likely to be useful for humans by a consensus conference of authorities convened by AGE (Meydani 1999). The biomarker data presented in this report demonstrate minimal free radical damage at supplemented levels of AA and AT. This is consistent with the hypothesis that higher

values for AT and AA reduce free radical damage *in vivo*. This study was conducted to demonstrate an effective design of a larger study to examine the effect of the combination of AA and AT at different dosages.

In conclusion, AT with low levels of AA protects lipid peroxidation and protein oxidation *in vivo*. For Heinz bodies, however, even when consuming 400 IU of AT daily, intake of 500 mg of AA twice a day alleviates peroxidative damage more effectively than 200 mg of AA in the diet. As AT is a fat-soluble antioxidant and AA is water-soluble, a study designed to examine the *in vivo* synergistic effects is likely to determine a reasonable combination of dosages. This pilot study demonstrates that even when 400 IU of AT is consumed, there is protective value of 500×2 of AA. Given the possible health benefits and widespread use of these antioxidants, thorough investigation of AT and AA in combination is warranted.

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