Design of a study to determine the Effect of Vitamin C and E intake on Protein carbonyls, TBARs and Heinz Bodies

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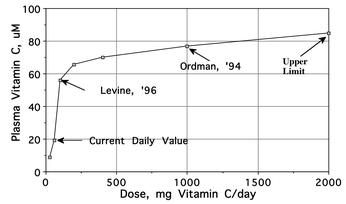
ABSTRACT

A clinical trial was conducted to compare the antioxidant effectiveness of three vitamin C dosages in the presence of 400 mg vitamin E. Dosages of vitamin C were 200 mg daily in food, 500 mg twice a day as supplements, and 1,000 mg twice a day as supplements, the Upper Limit established by the Food and Nutrition Board. Ten volunteers participated. Each spent 3 weeks at each dosage before blood samples were taken on two consecutive days, 4 -12 hours after supplementation. Participants were initially given instruction on portion size and vitamin C content of food and drinks available. Compliance was measured by food diaries and supplement time logs maintained during the days before blood draws.

Discussion of appropriate endpoints involved investigators at four institutions. Endpoints selected were protein carbonyls, TBARs, and Heinz body formation in RBCs. Protein carbonyls did not change significantly between the three vitamin C dosages. Even with daily vitamin E supplementation, Heinz body formation was significantly lower at 500 mg of vitamin C twice a day. Heinz body formation increased at either higher (200 mg) or lower (1,000 mg twice a day) intakes of vitamin C. Results indicate that even when vitamin E intake is 400 mg per day, it is possible to distinguish the effect of different levels of vitamin C on hemoglobin oxidation.

Figure 1: Effect of Twice Daily Oral Dose on Plasma Vitamin C Level



INTRODUCTION

Purpose: Many studies have begun appearing using vitamin C in human trials. This pilot study was conducted to highlight and calibrate important design features for a larger study. The need for combined supplementation studies has been noted recently(1), yet to be useful studies must report critical details like time between vitamin C intake and blood collection (2). In addition to determining useful dosage levels, we determined appropriate endpoints to measure antioxidant effectiveness in vivo. Even when vitamin E is taken at high levels, we expected to measure significant protection from oxidative damage as a function of vitamin C intake.

Dosage levels/Original studies: Beyond Dr. Linus Pauling's recommendation of up to 16 g of vitamin C per day, three "pharmacologic dosages" of vitamin C have been recognized. The first was 500 mg twice a day, the dosage at which vitamin C is continually excreted in the urine (3). The second was 200 mg in the diet, the dosage at which certain types of white blood cell are saturated with vitamin C (4). The third is the latest Food and Nutrition Board identification of an Upper Limit of vitamin C consumption (5). For oral consumption of vitamin C, either 500 or 1,000 mg twice a day results in statistically identical and maximal plasma levels. This study compared the effect of those three dosages.

Three methods of measuring free radical damage were selected based on discussion with colleagues.

TBARs: Thiobarbituric acid reactive substances assay is one method for measuring free radical generation. It is a frequently reported measure of antioxidant effectiveness.

Protein carbonyls: This is a relatively new method of higher sensitivity than TBARs. These products of free radical damage can now be conveniently assayed using a 96-well plate standard assay kit.

Heinz bodies: Heinz bodies in red blood cells (RBC) result from oxidative damage to hemoglobin. They have been shown to be especially sensitive for monitoring vitamin C effectiveness *in vivo*(6).

Figure 2: Dietary Regimens

Subjects spent 3 weeks per regimen in randomly-assigned order. Each completed all three regimens:

Regimen	Morning Vit. E	Morning Vit. C (sup.)	Evening Vit. C (sup.)	Diet	Total Vit. C
1	400 iu	none	none	trained to eat 200 mg in food	200 mg
2	400 iu	500 mg	500 mg	avoided vit C enriched drinks	500 mg twice a day
3	400 iu	1,000 mg	1,000 mg	avoided vit C enriched drinks	1,000 mg twice a day
Final	none	none	none	avoided vit C enriched drinks	minimal

METHODS

Beloit College IRB approval was received. Ten healthy participants from Beloit College volunteered for the study. The median group characteristics were 23 yr old, 178 cm, and 66 kg. All were healthy, though one smoked approximately 5 filtered cigarettes per day. Vitamin regimens and timeline are shown in Figures 2 and 3 resp. Blood samples were collected on two days at the end of each regimen.

For TBARs and protein carbonyls, serum was stored at -70oC until analysis. TBARs was determined by standard method (7) at the University of Arizona. Protein carbonyls were assayed with the Zentech PC Test (8) at the Linus Pauling Institute. For Heinz body counting, stained plasma smears were labelled in code and counted blind by three people before data were unblinded for analysis.

An ANOVA was performed using JMP to determine significance.

RESULTS

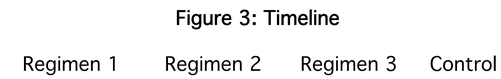
Neither TBARs nor protein carbonyl levels were significantly different from zero for any of the three regimens, nor following a week of vitamin depletion. It is likely that even after a week vitamin E levels were sufficient to remain protective.

In contrast, Heinz body was a useful relative measurement. There were significantly fewer with 500 mg of vitamin C taken twice a day. Figure 4 shows the number of Heinz bodies found in participants taking different levels of vitamin C. Except for the group labeled minimal, all were taking the vitamin C dosage shown and 400 iu of vitamin E daily. The highest level of Heinz bodies was in the group avoiding vitamins E and C, even though other endpoints indicate people retain substantial vitamin E. The group taking 1,000 mg of vitamin C twice a day had higher levels of Heinz bodies than those taking 500 mg twice a day.

In order to test the assumptions for an ANOVA, both Shapiro-Wilkes test for normality and Bartlett's test for equal variances were performed. As the assumptions were met, an ANOVA was performed, resulting in a significant difference among diet groups ($F = 6.4257$, total $df = 30$, $p = 0.002$). Subsequently, Dunnett's test was performed to ascertain the differences between the experimental groups and the control. The 500x2 ($q = 0.39010$) and 1000x2 ($q = 0.12706$) are significantly different from the control.

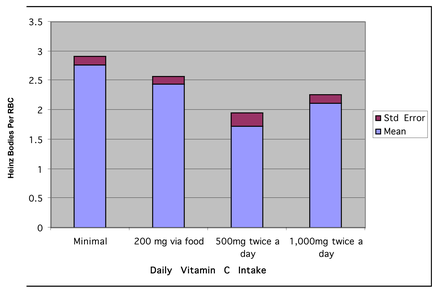
In Figure 5, data were analyzed controlling for individual differences. An individual first took 400 iu of vitamin E and the indicated vitamin C dosage daily for 3 weeks and Heinz bodies were counted. Then the individual avoided vitamins E and C for a week and blood was analyzed again. This way the change in Heinz bodies could be compared for individuals. This pilot study indicates 500mg twice a day produced fewer Heinz bodies than higher or lower doses of vitamin C.

Figure 3: Timeline



A. Avoided vitamin C enriched foods and all supplements
B. Randomly assigned to regimen 1, 2, or 3 for 3 weeks
C. On day 19 and 21, between 4-12 hours after morning supplements, blood was drawn by finger prick. Dietary logs were maintained for the four days ending with the last blood sampling. Continuous reminders were sent by email and telephone.
D. Vitamin C and E-rich foods and all supplements were avoided.
E. Two blood samples were taken 24 hours apart.

Figure 4: Pilot Study in presence of vitamin E: The effect of vitamin C intake on Heinz Body Formation in red blood cells *in vivo*.



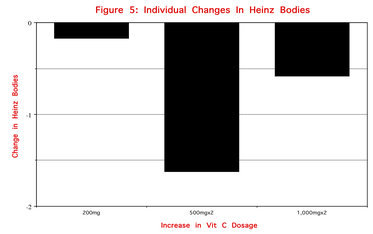
DISCUSSION

The study design demonstrated critical points for an effective study of antioxidants:

1. Time between intake of vitamin C and sampling must be carefully controlled between 4-12 hrs.
2. Different oxidative endpoints can measure synergistic or separate effects of water-soluble vit. C and fat-soluble vit. E.
3. Selection of vitamin C dosage should be justified based on prior studies with useful endpoint data.
4. A variety of convenient endpoints (urinary excretion, white blood cell saturation, plasma concentration, TBARs, protein carbonyls, Heinz bodies, arterial compliance) may measure different effects.

Vitamin E is dominant in suppressing TBARs and protein carbonyls. Even after a week of depletion, protection was still complete. Heinz body results are consistent with the hypothesis that 500mg of vitamin C taken twice daily may be substantially more effective in reducing hemoglobin damage. Figures 4-5 demonstrate that there may be significant disadvantage in consumption of 200 or 1,000x2 mg per day. The pro-oxidative effect of the higher dosage is similar to what has been observed in dosage comparison studies of vitamin E (9-10).

Figure 5: Individual Changes In Heinz Bodies



CONCLUSION

For TBARs and protein carbonyls, vitamin E predominates in protection in vivo. For Heinz bodies, however, even when taking 400 iu of vitamin E daily, consumption of 500 mg of vitamin C twice a day reduces peroxidative damage more effectively than 200 mg of vitamin C in the diet.

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