THESIS

VITAMIN C SUPPLEMENTATION: A COMPARISON OF DELIVERY METHODS AND THE ABILITY TO ATTENUATE OXIDATIVE STRESS INDUCED BY ISCHEMIA-REPERFUSION

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ABSTRACT

VITAMIN C SUPPLEMENTATION: A COMPARISON OF DELIVERY METHODS AND THE ABILITY TO ATTENUATE OXIDATIVE STRESS INDUCED BY ISCHEMIA-REPERFUSION

Intravenous delivery of vitamin C to adult humans decreases indices of oxidative stress and in some instances improves physiological function. Oral delivery of vitamin C is more practical than intravenous but typically results in lower circulating vitamin C concentrations. The hypotheses for this study were, oral consumption of vitamin C encapsulated in liposomes would: 1) result in higher circulating vitamin C concentrations than a traditional oral supplement, and 2) better attenuate oxidative stress induced by ischemia-reperfusion. Eleven overweight/obese adults [age: 52±7 years; body mass index: 34.1±1.0 kg/m²; mean±SE] were administered a 4 g supplement of placebo, or vitamin C via different delivery methods, on four separate occasions, in a random order. The four treatments were: placebo, oral vitamin C, liposomal vitamin C, and intravenous (IV) administration of vitamin C. Concentrations of ascorbic acid, thiobarbituric acid reactive substances (TBARS), and oxidized low-density lipoproteins (Ox-LDL) were measured in venous blood at baseline, and over four hours following supplement administration. At three hours a blood pressure cuff was placed around the upper arm and inflated to 200 mmHg for 20 minutes to evoke an ischemia-reperfusion injury. Plasma ascorbic acid concentrations were significantly greater after IV vitamin C compared with all other treatments at all time points (P<0.01). At two hours, all subsequent ascorbic acid concentrations were greater after liposomal vitamin C treatment compared with oral vitamin C and placebo treatments. Plasma ascorbic acid concentrations were greater after oral vitamin C compared with placebo (P<0.01). Neither vitamin C nor ischemia-reperfusion influenced Ox-LDL. In the placebo condition, ischemia-reperfusion increased plasma TBARS concentration; all of the vitamin C treatments prevented this increase. These data suggest that liposomal encapsulation of vitamin C increases bioavailability of oral vitamin C. Additionally, the
antioxidant protection provided by liposomal vitamin C is not inferior to intravenously administered vitamin C.
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CHAPTER I
LITERATURE REVIEW

Vitamin C in Disease Prevention and Treatment

Accumulating evidence suggests that vitamin C (ascorbic acid) may be an efficacious therapeutic agent in the treatment of several chronic diseases and conditions. Vitamin C treatment has been shown to decrease oxidative damage to lipids (1), lipoproteins (2) and deoxyribonucleic acid (DNA) molecules (3) and improve physiological function in humans. Reported physiological benefits of vitamin C supplementation have included enhanced vascular function in chronic smokers (4), and diabetic (5) and cardiovascular disease patients (3,6), improved regulation of blood pressure in hypertensive patients (7–9), and protection from ischemia-reperfusion induced endothelial dysfunction (3,10). However, some studies have reported no changes in oxidative stress biomarkers (11,12) and physiological function with vitamin C treatment (13–15).

Ascorbate (the anion of ascorbic acid) is a powerful reducing agent in biological systems. Ascorbate is responsible for donating electrons for enzymes involved in pro-collagen hydroxylation (16) and carnitine and norepinephrine biosynthesis (17). In addition, ascorbate is a strong water-soluble antioxidant in plasma and tissues, capable of scavenging reactive oxygen and nitrogen species (ROS and RNS) (18,19). Vitamin C also indirectly participates in antioxidative activities through its action in maintaining the vitamin E redox cycle (20).

Humans, unlike most mammals, have a mutation in the gene encoding gulonolactone oxidase, the rate limiting enzyme that catalyzes the conversion of L-gulonolactone into ascorbic acid (21,22); therefore, humans must obtain vitamin C in their diets. Humans deprived of ascorbic acid will develop scurvy (21), a disease related to vitamin C’s role in collagen synthesis. Daily vitamin C intake of ~45 mg is required to prevent scurvy (23), which is easily obtained without supplementation. However, due to the reported associations between vitamin C intake and protection from various diseases and conditions,
researchers (24,23,25) have advocated that larger amounts of vitamin C (90-200+ mg/d), beyond what is necessary to prevent scurvy, be consumed.

In the 1970s, Ewan Cameron and Linus Pauling (26) studied the therapeutic effects of vitamin C in cancer treatment. They based their hypotheses primarily on evolutionary observations. Animals, who can synthesize ascorbate, increase their production of vitamin C when they are diseased; thus, humans having lost the ability to synthesize vitamin C may require more in diseased states. They administered high-dose intravenous vitamin C (10g/d) to advanced cancer patients and reported a significant increase in survival time. This study established a basis to support the therapeutic use of large doses of vitamin C.

The Mayo Clinic reexamined Cameron and Pauling’s findings in subsequent randomized controlled trials with 10 g/d doses of oral vitamin C and found no difference in survival time between treated and untreated patients (27,28). Neither Cameron and Pauling, nor the Mayo Clinic provided plasma ascorbic acid concentrations with their findings, thus a pharmacological explanation could not be delineated (oral versus intravenous delivery). It has since been revealed that the bioavailability of vitamin C is tightly controlled and intravenous administration of vitamin C can achieve substantially greater plasma ascorbic acid concentrations than the same single dose administered orally (29).

Antioxidant Activity

Vitamin C effectively scavenges superoxide and other reactive oxygen species (30) and it plays an important role in the regulation of intracellular redox state (31). Vitamin C is capable of rapidly scavenging a number of ROS in both the extra and intracellular compartments. Intracellular ascorbate scavenges exogenous radical species that have entered cells or endogenous radicals produced as a result of excess superoxide generation by mitochondrial metabolism, NADPH oxidase, xanthine oxidase, or by uncoupled nitric oxide synthase (NOS) (32). It has been reported that scavenger activity of vitamin C is dependent on serum concentrations of 1 to 10 mmol/l or higher (33) to scavenge superoxide radicals (34). This provides evidence for the efficacy of supraphysiological concentrations of ascorbic acid.
Vitamin C also acts as a synergist to regenerate oxidized alpha-tocopherol (Vitamin E) (20), another powerful antioxidant. The vitamin E radical has the potential to attack polyunsaturated lipids and induce another chain oxidation. However, vitamin C reduces vitamin E radical to regenerate vitamin E before the vitamin E radical attacks lipids to induce lipid oxidation. Due to hydrophilic nature of ascorbic acid, it can scavenge aqueous radicals but is less efficient with scavenging lipophilic radicals in the membranes and LDL. Vitamin E complements the actions of vitamin C, as vitamin E’s primary function is to protect polyunsaturated fatty acids from oxidation (35). Thus, many antioxidant studies have administered a combined supplement with vitamin C and vitamin E (13,21).

Pro-oxidant

In vitro evidence suggests that vitamin C functions at low concentrations as an antioxidant but may have pro-oxidant activity at high concentrations. It remains to be proven whether vitamin C-induced reactive oxygen species occur in vivo. Ascorbic acid has been shown to reduce transition metals, such as cupric ions (Cu\(^{2+}\)), to cuprous (Cu\(^{1+}\)), and ferric ions (Fe\(^{3+}\)) to ferrous (Fe\(^{2+}\)) during conversion from ascorbate to dehydroascorbate in vitro. This reaction can generate superoxide and other ROS (36). The combination of ascorbic acid and iron is known and even used to induce free radical mediated oxidation in vitro. However, in the body, free transition elements are unlikely to be present while iron and copper are bound to diverse proteins. Thus, ascorbate as a pro-oxidant is unlikely to convert metals to create ROS in vivo. An extensive literature review by Carr and Frei (18) found limited evidence of a pro-oxidant effect of ascorbate on in vivo markers of DNA, protein, and lipid oxidation.

Pharmacokinetics and Bioavailability

In an effort to establish evidence-based vitamin C intake recommendations, The National Institutes of Health (NIH) conducted a series of pharmacokinetic experiments (37) in healthy human participants. Participants were first depleted to plasma vitamin C concentrations <8 µmol/L and then repleted with oral doses of vitamin C (30-2500 mg) twice daily in a fasted state. Doses were sequentially increased once steady state was reached (defined by at least 5 separate measurements of plasma concentrations over at least 1 week with <10% SD). At doses <100 mg/d, there was a steep sigmoid
relationship between dose and plasma concentration. At doses >100 mg/d, plasma concentrations reached a plateau between 70 and 80 µmol/L.

Maximal peak plasma concentrations, achieved with repeated oral dosing, are < 250 µmol/L, whereas plasma concentrations of >1,000 µmol/L are easily obtained by IV (29,38). Large doses given intravenously can result in maximum plasma concentrations of roughly 30 mM (39). The discovery that vitamin C may have poor bioavailability discredited many epidemiological studies that did not include measures of ascorbic acid concentrations in the blood (25,40). Many of these studies instead relied on dietary recall and falsely assumed that plasma ascorbic acid was reflective of dietary consumption.

**Intestinal Absorption**

Vitamin C distribution after oral delivery is tightly controlled by intestinal absorption, transport to tissues, and renal reabsorption and excretion (24). Vitamin C, consumed orally, is absorbed in the small intestine by active, sodium-dependent transport. The vitamin is transported as ascorbate into enterocytes via the Sodium-dependent Vitamin C Transporter 1 (SVCT1) (41). Transport of ascorbate across the basolateral membrane into circulation is likely a sodium-independent process via an unknown transporter (42). Sodium-dependent Vitamin C Transporter 2 (SVCT2) has a lower capacity but higher affinity for vitamin C than the SVCT1 isoform and is responsible for the uptake of ascorbate into most metabolically active cells and tissues. Due to its low molecular weight, vitamin C is freely filtered by the kidney, but reabsorbed in the renal proximal tubule, again by SVCT1.

When vitamin C intake is low, SVCT transporters in the kidney reabsorb ascorbic acid to prevent its loss by renal clearance and maintain ascorbic acid concentrations (43). Virtually zero renal clearance has been reported at ascorbic acid concentrations <45.4 µmol/L. The plasma half-life of ascorbic acid is 8-40 days with deficient intakes, providing the body with resistance against acute scurvy. When intake levels are higher, corresponding with plasma ascorbic acid >70µmol/L, ascorbate has a half-life of only ~30 minutes. Thus, the bioavailability of doses taken several hours apart should be considered independently; a large dose of vitamin C would be improved by dividing the dose into smaller ones taken a few hours apart.
Cellular uptake of oxidized form of ascorbate, dehydroascorbate, occurs via facilitated transport by the glucose transporters, GLUT-1 and GLUT-3. After uptake into the cell, dehydroascorbate is converted, by glutathione, to ascorbate, which by an unknown mechanism may be released into the extracellular space (44). This may serve as a protective function of regenerating ascorbate in individuals with inflammation. Thus vitamin C pharmacokinetics in diseased versus healthy individuals may be different.

**Toxicological Considerations**

High oral and intravenous doses of vitamin C appear to be safe for human use. Maximally tolerated oral doses are in the range of 3-4 g (45). However, high oral doses may cause gastrointestinal discomfort and mild diarrhea due to retention of ascorbate in the intestinal lumen and the osmotic water-retaining effect of unabsorbed vitamin C (46). In a Phase I clinical trial using intravenously administered vitamin C, minimal adverse events were reported in cancer patients receiving up to 1.5 g ascorbic acid/kg body weight, three times per week (47). In addition, intravenous vitamin C is commonly used by Complementary and Alternative Medicine practitioners (48) for the treatment of infection, cancer, and fatigue with few adverse events.

**Liposomal Encapsulation**

Liposomes are artificially made, microscopic, spherical vesicles composed of a lipid bilayer. Early research focused on creating liposomes to study lipid bilayers and the biology of biological membranes. A decade later, scientists realized the potential for liposomes to act as vehicles for the delivery of drugs and other small molecules. In 1995, the US Food and Drug Administration (FDA) approved Doxil, the first liposome drug delivery system, for human use (49). Doxil, liposome-encapsulated doxorubicin, is widely used to treat ovarian cancer and Kaposi’s sarcoma. Today, 15 liposome-encapsulated drugs are available for clinical use (49).

Liposomal encapsulation can alter both the tissue distribution and the rate of clearance of a drug by making the drug take on the pharmacokinetic characteristics of the carrier (50). The pharmacokinetic
properties of liposomal drugs are dependent upon the liposome, and not the parent drug, until the drug is released from the carrier (51).

Liposomes can be administered topically, intratracheally, intravenously, by inhalation, subcutaneously, by intramuscular injection or orally. Liposomal drug delivery systems have focused primarily on intravenous administration (49). However, oral dosing of liposomal pharmacological or nutraceutical delivery systems has advantages over invasive routes because of the potential increase in patient compliance and ease of use.

Emerging research has turned to liposomal delivery systems to enhance the bioavailability of nutraceuticals (52). Nanoencapsulation has been studied in the food industry to enhance the stability of encapsulated materials by protecting them from the environment, enzymes, extreme pH changes, temperature as well as masking unwanted odors or tastes (53). Ascorbic acid is unstable under heat and oxidation. Liposomal encapsulation has the potential to increase the stability and subsequently, the ‘shelf-life’ of vitamin C in foods and supplements. Encapsulating vitamin C inside liposomes has been proposed as a potential way to enhance its bioavailability (29).

Liposomes have the potential to carry both hydrophobic and hydrophilic compounds; the aqueous core can carry hydrophilic compounds, while lipophilic compounds can be carried within the hydrophobic region of the bilayer. Because liposomes are able to simultaneously contain (and deliver) both water- and lipid-soluble antioxidants, it has been suggested (54) that there may be a benefit to incorporating alphatocopherol (TOH) and ascorbate because as was mentioned earlier it has been demonstrated that ascorbate can regenerate TOH from the tocopheroxyl radical.

Resveratrol found in grapes, red wine, and peanuts has been studied as a pharmacological protective agent against several pathologies including cardiovascular disease, inflammation, cancer, obesity and diabetes. Most studies examining the beneficial health effects of resveratrol have been conducted in vitro and the human studies have been elusive (55). Resveratrol is characterized by poor bioavailability (56) which may stem from the compound’s low solubility in water, susceptibility to photodegradation, and rapid clearance from circulation (55,57). In recent years scientists have gained
interest in liposomal encapsulation of resveratrol to increase its stability and bioavailability (52); results have been promising. Liposomal encapsulation of resveratrol effectively protected it from light degradation (UV light-induced trans-cis isomerization) (58) and it was stable for up to three months at 4 degrees C. Resveratrol made with enriched soy phosphatidylcholine was superior to free resveratrol for protecting stressed cells in cell culture, and significantly increased antioxidative capability (59). Liposomal preparations have also increased resveratrol bioavailability. Administration of a liposomal resveratrol (soy phosphatidylcholine complex) to rats, via an enteral route, increased the levels of resveratrol ~2-fold in plasma and improved cardioprotective activity compared to free resveratrol (60).

Statement of the Problem

Supplementing humans with vitamin C has demonstrated improved physiological function in some studies but not in others. One of the reasons for the inconsistent results has been attributed to the poor bioavailability of orally administered vitamin C. Intravenous infusion bypasses the tight control offered by intestinal absorption and thus results in much higher plasma concentrations of vitamin C. However, intravenous infusion is not practical for the general population.

Liposomal encapsulation has been proposed as a potential way to enhance the bioavailability of vitamin C (29). However, research of liposomal encapsulation of vitamin C in humans is limited to a single pilot study (61). Therefore, we sought to compare the bioavailability of orally administered vitamin C encapsulated in a liposome with an un-encapsulated form and intravenously administered vitamin C.

Additionally, no studies have examined the efficacy of orally administered liposomal vitamin C in humans. One of the ways vitamin C improves physiological function is attributed to its role as an antioxidant. Therefore, we sought to quantify changes in circulating concentrations of biomarkers of oxidative stress before and after ischemia-reperfusion.

Hypotheses

Oral consumption of vitamin C encapsulated in liposomes will: 1) result in higher circulating vitamin C concentrations than oral consumption of vitamin C not encapsulated in liposomes, and 2) better attenuate oxidative stress induced by ischemia-reperfusion.
Specific Aims

(1) To compare the circulating concentrations of ascorbic acid following administration of vitamin C via three different delivery methods (oral; liposomal; intravenous) and a placebo. (2) To compare the changes in circulating concentrations of biomarkers of oxidative stress (TBARS; OxLDL) evoked by an ischemia-reperfusion injury, following administration of vitamin C via three different delivery methods (oral; liposomal; intravenous) and a placebo.
CHAPTER II
THE MANUSCRIPT

INTRODUCTION

Vitamin C (ascorbic acid), a powerful antioxidant, has been shown to decrease oxidative damage (62,44,3) and improve physiological function in humans. Reported physiological benefits of vitamin C have included enhanced vascular function in chronic smokers (4) and diseased patients (3,5,6), improved regulation of blood pressure (7–9), and protection from ischemia-reperfusion induced endothelial dysfunction (3,10). However, some studies have reported no changes in oxidative stress biomarkers (11,12) and physiological function following vitamin C supplementation (13–15). One of the reasons for the lack of consistent response to vitamin C treatment has been attributed to the poor bioavailability of orally administered vitamin C (43).

Vitamin C distribution after oral delivery is largely limited by intestinal absorption, in addition to being tightly controlled by transport to tissues, and renal reabsorption and excretion (24). Because blood is the transport medium for delivery of vitamin C to target tissues, plasma ascorbic acid concentrations are considered an appropriate indicator of vitamin C supply (63). When vitamin C is given by intravenous infusion, peak plasma concentrations over 10 mmol/L, can be attained (29,43) compared with peak plasma concentrations of <0.25 mmol/L with oral dosing (acute supplementation) (64). Intravenous administration is not practical for the general public, thus improving the bioavailability of orally administered vitamin C may be advantageous.

Liposomal encapsulation has been proposed as a potential way to enhance the bioavailability of vitamin C (29). Liposomes commonly used in the pharmaceutical industry (50) have improved the bioavailability of some antioxidants in vitro (54,60,65). The plasma concentration of coenzyme Q10 was ~2.2-fold higher when encapsulated in a soybean lecithin nanocapsule compared with a tablet form (65) and administration of liposomal resveratrol (soy phosphatidylcholine complex) not only increased the levels of resveratrol in plasma but also improved cardioprotective activity compared to free resveratrol.
A novel formulation of liposomal vitamin C has the potential to increase the molecular stability and may allow the vitamin to overcome transport limitations afforded by intestinal absorption. Thus, we hypothesize that oral consumption of vitamin C encapsulated in liposomes will result in higher circulating vitamin C concentrations than an equivalent dose of vitamin C not encapsulated in liposomes.

To demonstrate the efficacy of vitamin C at subsequent plasma concentrations the present study also aims to quantify the changes in circulating concentrations of biomarkers of oxidative stress following an oxidative injury. Ischemia followed by reperfusion results in a transient increase in the production of reactive oxygen species (10,66,67). In addition to increasing bioavailability, we hypothesize that liposomal vitamin C will better attenuate oxidative stress induced by ischemia-reperfusion compared with an equivalent dose of vitamin C not encapsulated in liposomes.

METHODS AND PROCEDURES

Experimental Design

A randomized, repeated measures, crossover design was used to compare placebo and three different delivery methods of vitamin C: oral vitamin C, oral vitamin C encapsulated in liposomes, and intravenous vitamin C. Subjects were administered all four treatments, each separated by at least seven days. Three hours after treatment administration, an ischemia-reperfusion injury was induced by a 20-minute upper arm occlusion. Venous blood was sampled at baseline and prior to and following ischemia-reperfusion to measure concentrations of vitamin C and concentrations of markers of oxidative stress in circulating plasma.

Subjects

Eleven adult humans (2 males and 9 females) participated in this study. Middle-aged and older adults (aged 45-70 years) who were overweight or obese (body mass index, BMI >25 kg/m²) were recruited because they were more likely to have lower levels of plasma vitamin C (68) and higher basal levels of oxidative stress than young, lean adults (69). Exclusion criteria included: regular use of vitamins, supplements or medications known to decrease oxidative stress during the previous three months, pregnant or nursing, history of allergic reaction or hypersensitivity to vitamin supplements, history of
kidney stones, and concurrent participation in another study. The experimental protocol was approved by the Colorado State University Institutional Review Board (Protocol #12-3790H) and followed the guidelines set forth by the Declaration of Helsinki. Each volunteer was informed of the purpose and potential risks of the study; written informed consent was obtained prior to enrollment.

Screening Visit

Subjects reported to the Human Performance Clinical Research Laboratory at Colorado State University for an initial screening visit before the start of treatments. The purpose of the screening visit was to collect health history information, anthropometric data, and familiarize subjects with the study protocol. Participants filled out a health history questionnaire. Height was measured to the nearest millimeter and body weight to the nearest 100 g using a stadiometer and beam scale (Detecto, Webb City, MO, USA). BMI was calculated as body mass/height² (kg/m²). Forearm blood-flow was occluded for four minutes to confirm that participants were tolerant to the rapid cuff inflator system (Hokanson, Bellevue, WA, USA) with the cuff positioned around the upper arm and pressure maintained at ~200 mmHg.

Treatment Visits

For each of the four treatment visits, participants reported to the laboratory following a 12-hour fast and 24 hours abstention from exercise. Participants relaxed in a semi-recumbent position for the duration of each visit (approximately 4.5 hours). A catheter was placed in an antecubital vein for blood sampling. After a baseline blood collection, subjects were administered one of the four treatments. A clock was started immediately following oral consumption of placebo (16 mL water), vitamin C, or liposomal vitamin C. For the intravenous vitamin C treatment, time began with the onset of a 60-minute vitamin C infusion, through the catheter already in place. Three hours after oral treatment (2 hours after intravenous), a blood pressure cuff was placed around the upper arm (proximal to the venous catheter) and inflated to 200 mmHg for 20 minutes. Blood samples (~10-15 mL) were obtained at minutes 60, 120, 180 (pre-cuff inflation), 200 (post-cuff inflation), 210, 220, 230 and 240.
**Vitamin C Preparation and Administration**

Oral vitamin C and liposomal vitamin C were hand delivered from Empirical Labs (Fort Collins, CO) in glass vials within a few days prior to participants’ scheduled visits. Vials were stored at room temperature and protected from light until use. A vial of oral vitamin C contained 4.25 g of 94% ascorbic acid (in powdered form); 16 mL of water was added to the vial and shaken vigorously prior to serving. Liposomal vitamin C, as formulated by Empirical Labs (Fort Collins, CO), contains 400 mg phosphatidylcholine per 1000 mg vitamin C (as sodium ascorbate; 125 mg sodium/1,000 mg ascorbic acid). Additional ingredients listed on the product label included purified water, natural flavors, and potassium sorbate (product code: LIPOSOMALVITAMINC). Study participants were administered a dose of liposomal vitamin C containing 4 g ascorbic acid and therefore, 1.6 g phosphatidylcholine and 500 mg sodium. Participants consumed the vial of liposomal vitamin C, followed by a rinse with 16 mL of water to match the 16 mL of water used to dissolve the powdered form of ascorbic acid described previously.

Ascorbic acid (500 mg/mL; Bioniche, USA) for the intravenous vitamin C treatment was stored at 2-8°C until use. Prior to infusion, the ascorbic acid was further dissolved in saline, such that 4 g of vitamin C were delivered in 100 mL of solution over 60 minutes. Intravenous vitamin C was infused using a Harvard Syringe Pump (Holliston, MA; rate=1.667 mL/min equivalent to 67 mg of vitamin C per minute) through the venous catheter already in place.

**Ischemia-Reperfusion**

Ischemia-reperfusion injury was induced by occlusion of forearm blood flow followed by reperfusion of forearm blood flow. A blood pressure cuff attached to a rapid cuff inflator system (Hokanson, Bellevue, WA, USA) was placed around the upper arm of each subject with the cuff maintained at a pressure of ~200 mmHg. After 20 minutes the cuff was deflated. Inflation of a cuff for 20 minutes followed by deflation of the cuff is an established model for creating oxidative stress in healthy humans (66,70).
Plasma Vitamin C

Blood samples (~5 mL) were collected in pre-chilled heparin tubes, placed immediately on ice and then centrifuged at 4°C and 3,600 rpm for 10 minutes to isolate plasma. Plasma samples (~1.5 ml) were transferred to opaque tubes to protect them from light and stored at -80°C until analysis. Plasma vitamin C concentration was measured via high-performance liquid chromatography with electrochemical detection by a commercial laboratory (LabCorp, Fort Collins, CO USA); laboratory personnel were blind to the treatments.

Lipid Peroxidation Measurements

Blood samples to be used for measuring markers of oxidative stress were collected in pre-chilled tubes containing ethylenediamine tetraacetic acid (EDTA), placed immediately on ice and centrifuged at 4°C and 3,600 rpm for 10 minutes to isolate plasma. Plasma samples were stored at -80°C until analysis. Enzyme-linked immunosorbent assays (ELISA) were used to measure, in duplicate, plasma concentrations of oxidized low-density lipoproteins (Ox-LDL; ALPCO Diagnostics, Salem, NH, USA). Plasma concentrations of malondialdehyde (MDA) were measured with thiobarbituric acid reaction using calorimetric detection (Cayman Chemical, Ann Arbor, MI).

Statistical Analysis

A two-way analysis of variance (treatment x time; ANOVA) with repeated measures was used to examine changes from baseline to post treatment within groups. Multiple comparisons of factor means were performed using Newman-Keuls test. The level of statistical significance was set at $P < 0.05$. Data are reported as mean ±SE.

RESULTS

Subjects

Eleven subjects completed all four treatment visits. All subjects were obese based on BMI. BMI and additional subject characteristics, including baseline plasma parameters (ascorbic acid and TBARS), are presented in Table 1. Three subjects were being treated for thyroid disease and two reported having high cholesterol. Two subjects were taking daily iron supplements and one was taking daily aspirin at the
time of the study; however, no drugs or supplements were consumed within 12 hours prior to treatment visits.

The rapid-cuff inflator and 20-minute ischemia followed by reperfusion was well tolerated by all subjects during all visits. One subject experienced muscle tightness and bruising from the distal edge of the cuff to their wrist following cuff deflation on one visit (cause unknown). Several subjects commented on the taste of the oral and liposomal vitamin C formulations. However, no adverse effects related to treatment were reported. One subject reported feeling relief from heartburn after the liposomal treatment.

*Plasma Ascorbic Acid*

Plasma ascorbic acid concentrations were significantly greater following intravenous administration of vitamin C compared to all other treatments at all time points \( (P < 0.001; \text{Figure 1A}) \). The magnitude of increase in circulating ascorbic acid concentrations following intravenous vitamin C administration was such that visual detection of differences between the remaining three treatments was difficult, thus these data have also been presented without the intravenous data (Figure 1B). At two hours, all subsequent ascorbic acid concentrations were greater in subjects with liposomal vitamin C treatment compared with oral vitamin C and placebo treatments and greater with oral vitamin C compared with placebo \( (P < 0.001; \text{Figure 1B}) \).

*TBARS*

Ischemia-reperfusion increased circulating thiobarbituric acid reactive substances (TBARS) concentrations in placebo; all vitamin C treatments prevented this increase (Figure 2). Specifically, TBARS concentrations were lower at 40 minutes in vitamin C compared with placebo. Plasma TBARS concentration did not differ between vitamin C treatments \( (P = 0.97) \). These data suggest the antioxidant protection provided by 4 g liposomal vitamin C is not inferior to 4 g intravenously administered vitamin C.
Oxidized Low-Density Lipoproteins

Neither vitamin C nor ischemia-reperfusion influenced circulating concentrations of oxidized-LDL ($P = 0.85$ and $P = 0.69$, respectively; Figure 3). The treatment and time interaction for oxidized-LDL was not statistically significant ($P = 0.932$).

DISCUSSION

The two major findings of this study are: (1) liposomal encapsulation of vitamin C increases bioavailability of oral vitamin C and (2) vitamin C treatment prevents the increase in circulating TBARS concentrations induced by ischemia-reperfusion.

Liposomal Vitamin C

Encapsulating vitamin C in liposomes increased plasma ascorbic acid by ~45% compared with oral consumption of the same amount of vitamin C not encapsulated in liposomes. Liposomal vitamin C formulations have been commercially available for some time despite the lack of scientific support for this method of administration in humans. Previous existing data on orally administered liposomal vitamin C in humans are limited to a single pilot study (61). In the aforementioned study, two subjects were orally administered 5 g vitamin C in phosphatidylcholine liposomes and a “standard formulation”. The plasma response curves and magnitudes of increase in plasma ascorbic acid concentrations were similar between the two formulations. With only two subjects studied there was likely insufficient statistical power to discern a difference (a type II error) as was observed in our study using eleven subjects.

In the present study, the peak plasma ascorbic acid concentration achieved by intravenous infusion was ~9 times less than that achieved by liposomal treatment, suggesting that liposomal encapsulation may improve but not completely overcome the bioavailability limitations afforded by oral consumption of vitamin C. When oral doses of vitamin C are increased, the bioavailability of the vitamin decreases, indicating that intestinal absorption contributes to tight control of plasma ascorbic acid concentrations (43,61). We would expect that transport of intact liposomes (carrying vitamin C) across the intestinal epithelium would result in similar concentrations of ascorbic acid in the plasma as those
achieved with intravenous delivery of vitamin. However, whether the liposomes unloaded the vitamin C before or after intestinal absorption is unknown.

*Antioxidant Activity of Vitamin C*

Evidence suggests that oxidative stress is a contributor to endothelial dysfunction and reperfusion injury in animals (71) and humans (10). The overproduction of ROS leads to consumption and depletion of endogenous antioxidants, including nitric oxide. Extremely reactive oxygen free radicals attack membrane phospholipids leading to the production of lipid peroxides, which disrupt the sarcolemmal integrity of cells. Due to their ability to scavenge ROS, the use of antioxidants is suggested in ischemia-reperfusion injury (10,66).

Vitamin C effectively scavenges superoxide and other reactive oxygen species (30). Lipid peroxidation products, measured as TBARS, are widely used as an indirect indicator of free radical activity. TBARS have been criticized as being a nonspecific marker of lipid peroxidation; however, experimental studies have shown good correlation between increases in TBARS and increases in levels of isoprostane (a potentially more specific marker of lipid peroxidation) in response to induced oxidative stress (72,73). In addition, TBARS was sensitive enough to be influenced by ischemia-reperfusion and vitamin C.

We observed no effect of ischemia-reperfusion or ascorbic acid treatment on circulating concentrations of Ox-LDL. In vitro evidence suggests that plasma concentrations of Ox-LDL may not peak until 24-hours after the start of reperfusion (74) and the present study captured only the first 40-minutes after the onset of reperfusion. Vitamin C protects LDL against oxidation by free radical scavenging or aqueous oxidants (75). As a water-soluble vitamin, ascorbate is not present in lipoproteins; therefore the major antioxidants protecting LDL against oxidation are lipid-soluble, such as alpha-tocopherol (21). Perhaps if intact liposomal vitamin C made it into circulation it would enter LDL and act as an antioxidant. However, plasma Ox-LDL concentrations may need to be examined for a period greater than four hours following vitamin C treatment administration to observe the effects.
Limitations and Experimental Considerations

Circulating concentrations of TBARS did not differ between vitamin C treatments despite the different plasma ascorbic acid concentrations achieved. Perhaps one of the reasons we did not observe a difference in scavenger capability is because of too small of a dose of vitamin C. A 4-gram dose was chosen for vitamin C because this is the highest dose of oral vitamin C (un-encapsulated) that can be tolerated. The upper limit vitamin C dose is 2 g and maximally tolerated oral doses are in the range of 3-4 g (76). A higher dose of IV vitamin C resulting in greater plasma ascorbic acid concentrations may have resulted in lower concentrations of lipid peroxides. The reason all vitamin C treatments may have decreased oxidative stress may be the result of plasma ascorbic acid concentrations surpassing a threshold. Suboptimal ascorbic acid has been proposed to be <50 µmol/L. In the present study, average baseline plasma ascorbic acid level was 0.84 mg/dL (47.7 µmol/L).

This study was the first to compare, in humans, administration of intravenous vitamin C, oral vitamin C, and oral vitamin C encapsulated in liposomes. The data suggests that liposomal encapsulation of vitamin C increases bioavailability of oral vitamin C and may have important clinical implications for treatment of disease.

TABLES

*After 12-hour fast, prior to treatment administration.

Table 1. Subject characteristics (n = 11).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SE</th>
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<tbody>
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<td>Sex (M/F)</td>
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<tr>
<td>Age (years)</td>
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<td>Height (m)</td>
<td>1.62 ± 0.02</td>
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<td>Body mass (kg)</td>
<td>89.0 ± 2.9</td>
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<tr>
<td>Body mass index (kg/m²)</td>
<td>34.1 ± 1.0</td>
</tr>
<tr>
<td>*Baseline Plasma Ascorbic Acid (mg/dL)</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>*Baseline TBARS (mM MDA)</td>
<td>5.82 ± 0.81</td>
</tr>
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</table>
Figure 1. Plasma concentrations of vitamin C (ascorbic acid) before (time = 0 min) and after treatment administration. Ischemia was induced immediately after the 60-minute plasma sample for 20 minutes. Data are reported with (A) and without (B) the intravenous treatment. *$P < 0.001$ vs. all other treatments; †$P < 0.001$ vs. oral; ‡$P < 0.001$ vs. placebo. Data are mean ± SE. Data suggest liposomal encapsulation of ascorbic acid may increase its bioavailability compared to an oral supplement of ascorbic acid not encapsulated in liposomes. Oral, 4 g ascorbic acid. Oral Lipo, 4 g ascorbic acid encapsulated in liposomes, consumed orally. IV, 4 g ascorbic acid infused intravenously over 60 minutes. Plasma ascorbic acid, 1 mg/dL = 56.78 µmol/L.
Figure 2. Vitamin C treatment attenuated the increase in circulating concentrations of thiobarbituric acid reactive substances (TBARS) following ischemia-reperfusion. #\(P < 0.046\) vs. all other treatments. \(\Delta\) = change from baseline. Data are mean ± SE. Oral, 4 g ascorbic acid. Oral Lipo, 4 g ascorbic acid encapsulated in liposomes, consumed orally. IV, 4 g ascorbic acid infused intravenously over 60 minutes. Baseline, prior to treatment. Inflate Cuff, 3 hours after baseline. Deflate cuff, after 20 minutes of ischemia. Post-Deflate, 40 minutes after deflation of cuff.
Figure 3. There was no effect of treatment or ischemia-reperfusion on circulating concentrations of oxidized low-density lipoprotein (LDL). Δ = change from baseline. Data are mean ± SE. Oral, 4 g ascorbic acid. Oral Lipo, 4 g ascorbic acid encapsulated in liposomes, consumed orally. IV, 4 g ascorbic acid infused intravenously over 60 minutes. Baseline, prior to treatment. Inflate Cuff, 3 hours after baseline. Deflate cuff, after 20 minutes of ischemia. Post-Deflate, 40 minutes after deflation of cuff.
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