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A Dose-Response Study on the Effects of Purified Lycopene Supplementation on Biomarkers of Oxidative Stress

Sridevi Devaraj, PhD, Surekha Mathur, PhD, RD, Arpita Basu, PhD, Hnin H. Aung, PhD, Vihas T. Vasu, PhD, Stuart Meyers, DVM, PhD, and Ishwarlal Jialal, MD, PhD

Laboratory for Atherosclerosis and Metabolic Research, University of California Davis Medical Center, Sacramento (S.D., S.M., A.B., S.M., I.J.), Department of Internal Medicine, University of California, Davis (H.H.A., V.T.V.), California

Abstract

Objective—While tomato product supplementation, containing antioxidant carotenoids, including lycopene, decreases oxidative stress, the role of purified lycopene as an antioxidant remains unclear. Thus, we tested the effects of different doses of purified lycopene supplementation on biomarkers of oxidative stress in healthy volunteers.

Methods—This was a double-blind, randomized, placebo-controlled trial, examining the effects of 8-week supplementation of purified lycopene, on plasma lycopene levels, biomarkers of lipid peroxidation {LDL oxidizability, malondialdehyde & hydroxynonenals (MDA & HNE), urinary F₂-isoprostanes}, and markers of DNA damage in urine and lymphocytes. Healthy adults (n = 77, age ≥ 40 years), consumed a lycopene-restricted diet for 2 weeks, and were then randomized to receive 0, 6.5, 15, or 30 mg lycopene/day for 8 weeks, while on the lycopene-restricted diet. Blood and urine samples were collected at the beginning and end of Week 2 of lycopene-restricted diet, and at end of Week 10 of the study.

Results—Independent of the dose, plasma lycopene levels significantly increased in all lycopene supplemented groups versus placebo (p < 0.05). ANOVA revealed a significant decrease in DNA damage by the comet assay (p = 0.007), and a significant decrease in urinary 8-hydroxy deoxoguanosine (8-OHdG) at 8 weeks versus baseline (p = 0.0002), with 30 mg lycopene/day. No significant inter- or intra-group differences were noted for glucose, lipid profile, or other biomarkers of lipid peroxidation at any dose/time point.

Conclusions—Thus, purified lycopene was bioavailable and was shown to decrease DNA oxidative damage and urinary 8-OHdG at the high dose.

Keywords

lycopene; DNA damage; oxidative stress; lymphocytes; lipid peroxidation; healthy subjects

INTRODUCTION

Lycopene is the most abundant carotenoid in tomatoes. Lycopene is the predominant circulating carotenoid in Western diets. Cancer and cardiovascular disease are the major cause of morbidity in North America. Increased lycopene levels have been associated with a

Address reprint requests to: I. Jialal, MD, PhD, Laboratory for Atherosclerosis and Metabolic Research, University of California Davis Medical Center, 4635 2nd Avenue, Research Bldg 1, Room 3000, Sacramento, CA 95817. E-mail E-mail: ishwarlal.jialal@ucdmc.ucdavis.edu.

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decreased risk of various types of cancer and cardiovascular disease [1,2]. Oxidative stress appears to play a pivotal role in the etiology of both cancer and atherosclerosis [3,4]. Lycopene, an acyclic symmetric hydrocarbon containing a high number of conjugated double bonds arranged linearly in the all-*trans* form, is the most potent singlet oxygen quencher among the natural carotenoids [5–7]. A systematic review of 72 epidemiological studies reported a consistent inverse relationship between intakes of tomatoes or plasma lycopene levels and prostate, lung, and stomach cancer [8]. Clinical studies have further demonstrated that dietary consumption of tomato products, containing lycopene, reduce biomarkers of oxidative stress (cellular DNA damage and biomarkers of lipid oxidation) in healthy subjects, smokers, and type 2 diabetics [9–13]. While effects on lipid peroxidation have been somewhat conflicting [14–16], several studies have consistently shown a significant decrease in lymphocyte DNA damage, following dietary intervention with tomato products in healthy human volunteers [12,17–19]. However, these studies do not suggest a role of lycopene per se, since tomato products contain several antioxidants, including carotenoids, lycopene, vitamins E and C and polyphenols, and each of these can interact to confer a preventive benefit against oxidative-stress associated diseases [20].

With respect to lipid peroxidation, *in vitro* studies have shown a superior capacity of tomato oleoresin over lycopene, in inhibiting *in vitro* LDL oxidation by up to five-fold. Purified lycopene has been shown to act synergistically with other natural antioxidants like vitamin E, the flavonoid glabridin, the phenolics rosmarinic acid and carnosic acid, and garlic, in inhibiting LDL oxidation *in vitro* [21].

While results from *in vitro* experiments, epidemiological studies, and clinical trials, report that tomato carotenoids may be powerful antioxidants *in vivo*, there is scanty data following supplementation with purified lycopene per se in humans. In this dose-response study, we investigated the effects of purified lycopene supplementation (0, 6.5, 15, 30 mg/day) on biomarkers of oxidative stress in healthy human volunteers.

SUBJECTS AND METHODS

Study Design

This was a double-blind, placebo-controlled intervention trial; the study design was approved by the Institutional Review Board (IRB) of the University of Texas Southwestern Medical Center at Dallas and University of California Davis Medical Center at Sacramento. Apparently healthy subjects with mildly elevated cholesterol levels (200–240 mg/dL), were included in the study. The subjects provided informed consent to participate in the study. Eighty-two healthy adults ≥ 40 years of age with plasma oxygen radical absorbance capacity (ORAC) activity less than 75th percentile of the normal population ($<3.7 \mu\text{M}$ Trolox equivalents as set up in the PI's laboratory) were enrolled in this study. Subjects were excluded if they smoked or consumed more than 30mL of alcohol per day, took antioxidants or fish oil supplements, had any history of chronic disease like diabetes, hypertension, heart disease, renal disease; had an abnormal blood chemistry profile; or were taking hypolipidemic medications. All subjects had fasting cholesterol concentration < 240 mg/dL, or fasting triacylglycerol concentration < 200 mg/dL, at the time of the screening.

To standardize their diets, all subjects maintained a two-week acclimation period before the initiation of the study, when they took a placebo (one capsule/day) and refrained from all lycopene-containing foods. The subjects were given printed guidelines on foods that were to be avoided, including, tomato and tomato products, watermelon, pink guava, papaya, and pink grapefruit, which are rich sources of lycopene [22].

Following the two-week acclimation period, the subjects were randomly assigned to receive placebo, 6.5 mg, 15 mg, or 30 mg/day of lycopene capsules for eight weeks, while continuing to follow the lycopene-restricted diet. Our rationale for dose selection (6.5, 15, or 30 mg lycopene) is based on previous studies, which have shown that lycopene supplementation at these doses, present in tomato products, is bioavailable, as well as protective against oxidative stress. All subjects kept a food diary. The lycopene capsules were supplied by DSM Nutritional Products, Inc. of Parsippany, NJ as redivivo (tm) WS, a 10% w/w water soluble beadlet. The capsules were composed of synthetic crystalline lycopene (all-*trans*) and were packaged in two-piece hard-shell capsules mixed with lycopene-free beadlets in the following combinations: 6.5 mg lycopene (65 mg lycopene 10% WS, 335 mg lycopene-free beadlets), 15 mg lycopene (150 mg lycopene 10% WS, 250 mg lycopene-free beadlets), and 30 mg lycopene (300 mg lycopene 10% WS, 100 mg lycopene-free beadlets). Ascorbyl palmitate (5% by weight) and dl-alpha-tocopherol (1.5% by weight) were added to the supplements/placebo as antioxidants to stabilize the crystalline lycopene. The capsules were packaged in white polypropylene bottles with a foil seal to keep out oxygen and light. The subjects were asked to consume one capsule per day with low fat milk. Compliance was assessed at the end of the study using the method of pill counting and measurement of plasma lycopene levels.

All blood samples were collected following an overnight fast (10–12h). Blood and urine samples were collected prior to lycopene restriction (Visit A), after 2 weeks of lycopene-restricted diet or baseline (Visit B), and at 8 weeks (Visit C) following placebo or lycopene supplementation. Serum and plasma (Heparin and EDTA) were separated from blood cells by low-speed centrifugation (1000×g for 10 min) at 4°C. The samples were then aliquoted, purged with nitrogen, and stored at –80°C for measurement of plasma lycopene levels, and biomarkers of lipid peroxidation (LDL oxidizability, MDA & HNE) at the conclusion of the study. Freshly separated serum was used for determination of the lipoprotein profile and glucose concentrations. Lymphocytes were separated by density gradient centrifugation with Histopaque 1077 (Sigma Chemicals Co, St Louis, MO, USA). The lymphocyte layer was removed from the gradient, washed with RPMI and resuspended in 1 ml of ice cold PBS. The freshly prepared cells were then treated with 100µM H₂O₂ for 10 minutes for determination of DNA damage using the Comet assay. All samples were numbered consecutively so that sample identity and treatment assignment were not apparent. Furthermore, the laboratory staff were blinded to the treatment assignments.

24-hour urine samples were centrifuged, flushed with nitrogen, and stored at –80°C for determination of F₂-isoprostanes and 8-OHdG at the conclusion of the study.

Analytic Procedures

Serum glucose, lipids, and lipoprotein concentrations were determined, and safety parameters, including kidney, thyroid, and liver functions tests and a complete blood cell count (CBC) were assayed using standard laboratory techniques. Lycopene extraction from plasma samples was performed according to a modified method by Obermuller-Jevic et al. [23] using high-performance liquid chromatography (HPLC) using the Waters Breeze Chromatography software (version 3.20, Waters Corporation, MA). Recovery rates were between 75% to 85%. The within- and between-days variability for lycopene measurement by our method was 5.5% and 8.4%, respectively.

LDL oxidation rate and lag time were measured by monitoring the formation of conjugated dienes after *in vitro* Cu²⁺-catalyzed oxidation as described previously [24]. Lipid peroxidation was measured in plasma as malondialdehyde (MDA) and 4-hydroxynonenal (HNE), using a colorimetric assay according to manufacturer's instructions (LPO-586™, Oxis Health Products, Inc., Portland, OR) based on the reaction of MDA or 4-HNE with a chromogenic reagent to yield a stable chromophore with maximal absorbance at 586nm.

F₂-Isoprostanes were measured in 24-hour urine samples with the use of a previously described enzyme immunoassay (EIA) method (Cayman Chemicals, Ann Arbor, MI) [24]. We previously validated this method against gas chromatography-mass spectrometry [25]. Urinary 8-OHdG was quantitated using a competitive enzyme-linked immunosorbent assay (Oxis Health Products, Inc., Portland, OR). F₂-Isoprostanes and 8-OHdG were standardized to urinary creatinine measured by standard techniques.

Susceptibility of DNA against oxidative challenge was assessed by the Comet assay using the TREVIGEN® Comet-Assay™ Silver (R&D Systems, Minneapolis, MN) as per manufacturer's protocol. Briefly, lymphocytes were treated with 100μM H₂O₂ for 10 minutes at 4°C. The normal and treated cells were combined with molten low melting point agarose, pipetted onto the Comet slides, which were then placed flat at 4°C in the dark for 30 minutes for the development of a 0.5mm clear ring at the edge of Comet slide area, following which they were immersed in pre-chilled Lysis solution at 4°C for 30 minutes, then in freshly prepared Alkaline Unwinding solution, pH > 13 for 30 minutes at room temperature in the dark. The slides were then removed, washed and then subject to electrophoresis using 1X TBE buffer. Silver staining was performed following fixation. Individual cells or "comets" were viewed at a magnification of 40X under a fluorescence microscope (BX60; Olympus Italia, Milan, Italy), attached to a high-sensitivity CCD videocamera (Zeiss Axio Cam, MB, Canada), and to a computer with an image analysis system. The comet tails (control and treated cells) were quantified using the NIH software available at <http://rsb.info.nih.gov/nih-image>. The Inter-assay coefficient of variation of the assay was < 11%.

Statistical Analysis

A repeated measures two-factor analysis of variance was performed to test the effects of different supplemental doses of lycopene at different time points, or their interaction, on different variables of interest. When significant interactions were observed, pair wise comparisons were made within groups by using 2-sample *t* tests or Wilcoxon rank-sum tests. Data are presented in the text and tables as means ± SDs. Significance was defined as *p* < 0.05. Statistical analyses were conducted with SAS software (version 8.2; SAS Institute Inc, Cary, NC).

RESULTS

While 82 subjects entered the study, 5 dropped out and 77 subjects completed the study. Out of 5 dropouts, one subject in the placebo group, and another subject in the group supplemented with 15 mg lycopene/day, complained of allergic skin reactions, while the other 3 subjects discontinued the study for personal reasons. None of the subjects complained of bloating, diarrhea or other gastrointestinal problems. Thus, compliance was high (93.9%) and no serious adverse reactions due to purified lycopene supplementation were noted among the subjects who completed the study.

Table 1 shows the salient characteristics of the study subjects. There were no significant differences between the groups at any of the Visits. Lycopene supplementation at any dose, did not affect any of the safety parameters in subjects, including liver, kidney and thyroid function tests and CBC (data not shown). Furthermore, there was no significant effect on the lipoprotein profile.

Plasma lycopene concentrations were significantly reduced in all four groups, following the two-week lycopene-restricted diet (Visit B or baseline), compared to Visit A (prior to the lycopene restricted diet) (*p* < 0.05). Lycopene supplementation resulted in increased plasma all *trans* lycopene levels in each supplemented group compared to baseline but not placebo at

8 weeks (Visit C) (Fig. 1, $p < 0.05$). Lipid standardized lycopene levels also showed similar changes as plasma lycopene levels (data not shown).

Biomarkers of Oxidative Stress

As shown in Table 2, LDL susceptibility to oxidation, assessed by lag time after copper-induced oxidation, and plasma MDA and HNE levels were not modified by lycopene supplementation. Similarly, no effects were observed on urinary F₂-isoprostane levels in any of the supplemented groups. However, repeated measures ANOVA revealed significant effects of time only on lymphocyte DNA damage ($p = 0.007$), in subjects receiving 30 mg lycopene/day. The comet assay showed a significant 8.9% decrease in comet tail lengths, indicating decreased lymphocyte DNA damage at 8 weeks (Visit C) versus baseline (Visit B) in the 30 mg/day group (Fig. 2, $p < 0.01$). Since we observed a significant effect of 30 mg lycopene supplementation in reducing DNA damage by the Comet assay, we further assessed urinary 8-OHdG, another biomarker of DNA damage in placebo and 30mg/day lycopene groups at baseline and 8 weeks. Repeated measures ANOVA further showed significant effects of time only on urinary 8-OHdG ($p = 0.0002$) in the same group. Urinary 8-OHdG concentrations showed a significant 23% reduction at 8 weeks (Visit C) versus baseline (Visit B) with 30 mg/day lycopene supplementation, but not with placebo (Fig. 3, $p < 0.01$).

DISCUSSION

In this randomized, double-blind, placebo-controlled, dose-response study, purified lycopene has been shown to exert beneficial effects on biomarkers of oxidative stress, particularly in decreasing DNA oxidative damage. Our rationale for dose selection (6.5, 15, or 30 mg lycopene) is based on previous studies, which have shown that lycopene supplementation at these doses, present in tomato products, is bioavailable, as well as protective against oxidative stress [26,27]. Furthermore, these doses are achievable through diet, and the range also conforms to the daily recommended levels of lycopene intake [28].

In the present study, independent of the dose, purified lycopene supplementation significantly increased plasma lycopene levels in all three groups, in comparison to baseline (Visit B) and placebo. The significant decrease in plasma lycopene levels at Visit B versus Visit A (prior to lycopene restriction), in all four groups, confirms the adherence of the subjects to the lycopene-restricted diet for 2 weeks. Plasma lycopene concentrations did not increase at 8 weeks (Visit C) in the placebo group. The lycopene-supplemented groups (6.5, 15, or 30mg lycopene), showed significant increase at Visit C compared with baseline, confirming the compliance of the study subjects and the bioavailability of synthetic, purified lycopene supplementation. Limited data exists regarding the bioavailability of synthetic lycopene in humans. Hoppe et al. [29], reported that a 28-day supplementation of 15 mg/day of synthetic lycopene (Lycovit 10% beadlets), or tomato-based lycopene (Lyc-O-Mato), led to identical bioavailability in healthy normolipidemic subjects. In a recent study, Zhao et al. [30] also showed a significant increase in plasma lycopene levels (from 0.5 $\mu\text{mol/L}$ on day 1 to 1.5 $\mu\text{mol/L}$ on day 57) in healthy post-menopausal women, following a 56-day supplementation of 12 mg/day of synthetic lycopene. The present study shows the bioavailability and tolerability of synthetic lycopene supplementation at three different dietary achievable doses of 6.5, 15, and 30 mg/day.

Several studies have shown the beneficial effects of tomato products, or other carotenoid-rich vegetable (spinach, carrot) consumption, in reducing lipid peroxidation in adults [13,31–34]. Some of these studies did not have a wash out period between dietary interventions, to closely mimic the dietary behavior of consumers, while the others reported an increase in plasma levels of tomato carotenoids, like β -carotene, phytoene, phytofluene, in addition to lycopene. Thus, the beneficial results postulated by these studies do not support the exclusive role of lycopene as the beneficial agent in reducing lipid peroxidation. Our observations of the null effects of

lycopene supplementation on 2 biomarkers of lipid peroxidation (LDL oxidizability and urinary F₂-isoprostanes; Table 2) suggest that combined carotenoids present in food compared to those of an isolated antioxidant supplement, i.e., purified lycopene, may be beneficial in protecting against lipid oxidation.

An important objective of this present study was to verify whether the increase in plasma lycopene concentration correlated with improvement in cellular defenses against oxidative stress. We found that a daily dose of 30 mg lycopene was potent enough to produce an approximately 9% reduction in DNA damage with regard to baseline levels, suggesting a beneficial effect of a moderate dose of lycopene supplementation on a surrogate marker of mutagenesis (Fig. 2). Previous observations have reported up to 50% lymphocyte DNA protection, following tomato puree supplementation, providing about 7 or 16 mg lycopene/day [17,18]. Zhao et al. [30] further reported that a supplementation of 12 mg/day of synthetic lycopene for 56 days in 37 healthy non-smoking postmenopausal women showed a significant reduction in DNA damage in lymphocytes compared to baseline. Our study findings show similar effects in subjects supplemented with 30mg lycopene/day, and this difference in dose-response effects may be attributed to lower levels of baseline plasma lycopene in the present study population, compared to the values reported by Zhao et al. [30] in postmenopausal women or the biological differences in populations between the 2 studies. It is also possible that subjects with low baseline lycopene levels respond better to low doses of lycopene than subjects with elevated baseline levels of lycopene. Another important biomarker of DNA damage which was noted to be affected by lycopene supplementation at 30 mg/day was urinary 8-OHdG. Increased 8-OHdG have been observed in target tissues of several animal cancer models, and in human leukocytes from patients with various diseases associated with oxidative stress [35,36]. Bowen et al. [37] observed a significant decrease in leukocyte 8-OHdG in patients with localized prostate adenocarcinoma who consumed tomato sauce-based pasta dishes for 3 weeks, providing about 30 mg lycopene/day. However, our study shows reduction of urinary 8-OHdG concentrations in subjects supplemented with 30 mg purified lycopene/day, revealing the antioxidant role of lycopene per se. The beneficial effects observed at this dose may also be explained by the hypothesis mentioned in a recently published Executive Summary on lycopene, which states that doses equal to 30 mg or higher may remain in the intestine for a longer time, than lower doses [38]. However, we did not test 8-OHdG at lower doses of lycopene and this needs to be tested in future studies.

In summary, lycopene supplementation at all doses were bioavailable, but did not affect biomarkers of lipid peroxidation in plasma and urine, at least at doses used in our study (6.5, 15, or 30 mg/day) in healthy human adults on a lycopene restricted diet. However, our data shows the exclusive role of lycopene as a cellular antioxidant in reducing lymphocyte DNA susceptibility against oxidative damage and urinary 8-OHdG, in healthy subjects consuming a daily supplement of 30 mg purified lycopene. These effects of lycopene may have important consequences in the prevention of prostate, lung, and stomach cancers, as reported by previous epidemiological observations and warrants further investigations with larger clinical trials.

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Fig. 1.

Plasma Lycopene levels in subjects before and after supplementation: Plasma lycopene levels were measured prior to lycopene restriction (Visit A), baseline (Visit B-following lycopene restricted diet) and at 8 weeks of the study (Visit C-end of supplementation) by HPLC as described in Methods. Data are given as Mean ($\pm$ SD) in the 4 groups (placebo, 6.5, 15, or 30 mg/day). * $p < 0.05$ at Visit B vs. Visit A in all groups; # $p < 0.05$ at Visit C vs Visit B, and placebo.”

Fig. 2.

Effect of Lycopene supplementation on Lymphocyte DNA damage: Lymphocyte DNA damage was assessed by measurement of comet tail lengths in healthy subjects supplemented with placebo, 6.5, 15, or 30 mg lycopene/day for 8 weeks as described in Methods. Data are presented as Mean ($\pm$ SD) of comet tail lengths (μ m). Visit A-Prior to Lycopene restricted diet; Visit B-following lycopene restricted diet and Visit C-end of supplementation * $p < 0.05$ at 8 weeks vs. baseline in the 30 mg lycopene/day group.”

Fig. 3.

Effect of Lycopene supplementation on 8-OHdG: 8-OHdG levels were measured in urine of healthy subjects supplemented with placebo or 30 mg lycopene/day for 8 weeks as described in Methods. Data are presented as Mean ($\pm$ SD) of 8-hydroxy-2'-deoxyguanosine (8-OHdG; ng/mg creatinine). * $p < 0.05$ at 8 weeks vs. baseline (Visit C vs Visit B)."

Table 1

Subject Characteristics, Glucose and Lipid Profile Before Lycopene Restricted Diet (Visit A), Baseline (Visit B-folllowing lycopene restriction) and End of Treatment (Visit C) (Mean $\pm$ SD)

Variable	Placebo n = 18	Lycopene 6.5 n = 21	Lycopene 15 n = 17	Lycopene 30 n = 21
Age (years)	49.9 $\pm$ 15.8	51.1 $\pm$ 5.9	49.8 $\pm$ 8.2	51.2 $\pm$ 9.7
Male/Female Ratio	4/14	4/17	4/13	7/14
BMI (kg/m ²)	28.1 $\pm$ 9.8	29.6 $\pm$ 5.2	25.4 $\pm$ 3.6	26.4 $\pm$ 6.1
Serum Cholesterol (mg/dL)				
Visit A	208.2 $\pm$ 71.9	203.8 $\pm$ 25.6	197.6 $\pm$ 20.2	194.6 $\pm$ 20.4
Visit B	205.7 $\pm$ 68.3	205.1 $\pm$ 27.1	207.3 $\pm$ 22.4	199.2 $\pm$ 22.5
Visit C	204.7 $\pm$ 30.2	203.3 $\pm$ 27.2	210.4 $\pm$ 19.9	201.5 $\pm$ 20.4
LDL Cholesterol (mg/dL)				
Visit A	124.8 $\pm$ 30.9	127.9 $\pm$ 22.8	120.0 $\pm$ 21.6	120.9 $\pm$ 19.5
Visit B	124.7 $\pm$ 24.6	129.4 $\pm$ 19.4	131.4 $\pm$ 19.3	121.4 $\pm$ 24.7
Visit C	123.9 $\pm$ 27.0	125.1 $\pm$ 21.3	125.4 $\pm$ 21.4	123.4 $\pm$ 23.0
HDL Cholesterol (mg/dL)				
Visit A	56.8 $\pm$ 9.2	54.4 $\pm$ 13.9	64.8 $\pm$ 14.3	56.5 $\pm$ 19.8
Visit B	56.7 $\pm$ 9.2	55.5 $\pm$ 11.2	62.6 $\pm$ 13.2	56.5 $\pm$ 17.8
Visit C	56.9 $\pm$ 10.2	56.2 $\pm$ 10.9	66.7 $\pm$ 15.7	55.8 $\pm$ 16.8
Serum Triacylglycerol (mg/dL)				
Visit A	94.8 $\pm$ 27.4	91.1 $\pm$ 36.6	88.1 $\pm$ 24.3	107.5 $\pm$ 45.9
Visit B	99.6 $\pm$ 40.8	87.7 $\pm$ 36.9	87.2 $\pm$ 23.8	100.7 $\pm$ 45.8
Visit C	103.5 $\pm$ 44.5	95.9 $\pm$ 36.3	90.1 $\pm$ 39.4	100.9 $\pm$ 45.6
Serum Glucose (mg/dL)				
Visit A	91.5 $\pm$ 7.7	90.5 $\pm$ 11.4	90.7 $\pm$ 7.0	97.3 $\pm$ 14.2
Visit B	89.8 $\pm$ 9.1	92.9 $\pm$ 10.8	95.8 $\pm$ 8.5	97.5 $\pm$ 14.8
Visit C	94.2 $\pm$ 8.00	90.4 $\pm$ 11.0	88.3 $\pm$ 9.4	99.9 $\pm$ 15.6

No significant effects of time or treatment ($p > 0.05$)

Table 2
Biomarkers of Oxidative Stress in Plasma and Urine of Subjects Assigned to Receive Placebo, 6.5, 15, or 30 mg Lycopene/Day before Lycopene Restricted Diet (Visit A), Baseline (Visit B-Following Lycopene Restriction) and End of Treatment (Visit C) (Mean $\pm$ SD)

Variable	Placebo n = 18	6.5 mg lycopene n = 21	15 mg lycopene n = 17	30 mg lycopene n = 21
LDL lag time (min)				
Visit A	67.8 $\pm$ 11.3	64.0 $\pm$ 9.80	65.6 $\pm$ 9.40	70.6 $\pm$ 9.1
Visit B	67.8 $\pm$ 11.6	66.1 $\pm$ 12.7	65.4 $\pm$ 9.9	72.5 $\pm$ 9.9
Visit C	69.0 $\pm$ 15.6	67.6 $\pm$ 13.6	63.7 $\pm$ 12.8	68.6 $\pm$ 18.0
LDL oxidation rate (nmol/min)				
Visit A	3.83 $\pm$ 1.8	4.59 $\pm$ 2.04	4.42 $\pm$ 2.15	3.74 $\pm$ 1.64
Visit B	4.03 $\pm$ 1.7	4.35 $\pm$ 1.81	4.67 $\pm$ 1.79	4.05 $\pm$ 1.66
Visit C	3.68 $\pm$ 1.6	4.17 $\pm$ 1.76	4.04 $\pm$ 1.89	3.80 $\pm$ 1.70
Lipid peroxidation (MDA & HNE μ M)				
Visit A	1.29 $\pm$ 0.88	1.27 $\pm$ 0.81	1.19 $\pm$ 0.68	1.32 $\pm$ 0.87
Visit B	ND	ND	ND	ND
Visit C	1.47 $\pm$ 1.03	1.16 $\pm$ 0.92	1.02 $\pm$ 0.61	1.19 $\pm$ 0.61
Urinary F ₂ -isoprostanes (pg/mg creatinine)				
Visit A	707.7 $\pm$ 489.0	535.7 $\pm$ 233.3	502.9 $\pm$ 274.6	615.6 $\pm$ 435.5
Visit B	656.2 $\pm$ 388.9	557.1 $\pm$ 241.2	490.3 $\pm$ 288.3	612.3 $\pm$ 412.1
Visit C	641.4 $\pm$ 347.9	474.9 $\pm$ 195.2	427.8 $\pm$ 257.9	587.6 $\pm$ 433.2

No significant effects of time or interaction ($p > 0.05$). ND = not done.