ABSTRACT

Title of thesis: Chronic Ingestion of (3R,3'R,6'R)-Lutein and (3R,3'R)-Zeaxanthin in Female Rhesus Macaque Primates

Edra London, Master of Science, 2006

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Age-related macular degeneration (AMD) is the leading cause of blindness in developed countries in individuals over the age of 65. High intake of the dietary carotenoids lutein (L) and zeaxanthin (Z) is believed to reduce the risk of AMD. This study investigated the effects of long-term supplementation of primates with high doses of L or Z, and their 1:1 combination, and whether high supplemental doses cause ocular toxicity.

Eighteen female rhesus macaques were divided into four groups: control (n=3), L-treated (n=5, 9.34 mg/kg L and 0.66 mg/kg Z), Z-treated (n=5, 10 mg/kg Z), and L/Z-treated (n=5, L and Z each at 0.5 mg/kg). At 6 month intervals beginning at baseline, plasma samples were analyzed by HPLC for L, Z, and their metabolites. Carotenoid analysis of tissues, ocular examinations, and toxicity assays were performed.

High-dose supplementation of primates with L or Z significantly increased plasma, and ocular and other tissue concentrations of these carotenoids and their metabolites in most cases. Supplementation with a 1:1 dose of L and Z increased plasma concentrations of these carotenoids after 6 months, but baseline and month 12 levels in plasma and ocular tissues were not significantly different. Supplementation of primates with L or Z at high doses does not cause ocular or kidney toxicity.

CHRONIC INGESTION OF (3R,3'R,6'R)-LUTEIN AND (3R,3'R)-ZEAXANTHIN IN FEMALE RHESUS MACAQUE PRIMATES

by

Edra London

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2006

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ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Frederick Khachik, for his support and guidance throughout my studies in the Master's Program in Nutrition. His expertise in the field of carotenoids and in conducting laboratory research as well as his advice on the preparation of my data and this thesis was invaluable. I would also like to thank the other members of my graduate advisory committee, Dr. Mark A. Kantor and Dr. Liangli Yu for their valuable feedback regarding the organization of my data, clarification of the study design, and for their other useful suggestions for improving this thesis. Dr. Bernadene Magnuson, originally a member of my graduate committee, also provided much thoughtful input during the initial stages of this project. Special acknowledgment is due to Dr. Fabiana de Moura who provided much of the statistical analysis for this study, and who patiently taught me about carotenoid extraction and HPLC analysis upon my joining Dr. Khachik's laboratory group.

This study was funded by the National Eye Institute of the National Institutes of Health. Without their financial support, this project would not have been possible. Thanks are also due to DSM Nutritional Products Ltd., Human Nutrition and Health Division, Basel, Switzerland for providing the beadlets of lutein and zeaxanthin that were used in this study.

Additionally, I would like to thank my fellow graduate students in the Department of Nutrition and Food Science for their ongoing support, and my lab mate An-Ni Chang for her thoughtfulness and her technical expertise in the laboratory which she so generously shared.

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INTRODUCTION

Age-Related Macular Degeneration (AMD) is the most common cause of blindness and visual impairment in Americans age 60 or older [National Eye Institute (NEI), 2004]. NEI and the Eye Disease Prevalence Research Group estimate that 1.8 million Americans currently suffer from AMD-related vision loss and that number will rise to a projected 2.9 million by the year 2020. The exact etiology of AMD is currently unknown.

AMD is characterized by the degeneration of the retina and the retinal pigment epithelium (RPE) in the macular region that causes progressive vision loss. The macular region is characterized as the small depression in the center of the retinal surface called the fovea, and the presence of yellow pigments that accumulate there. This area is extremely rich in cone photoreceptors that are responsible for enabling maximal visual acuity.

It was not until the publication of a case-controlled epidemiological study by Seddon et al. in 1994 that increased attention was drawn to AMD. This study revealed that high consumption of fruits and vegetables, specifically those rich in two dietary carotenoids, lutein and zeaxanthin, reduced the risk of AMD (Seddon et al., 1994). High consumption was defined as approximately 6 mg/day of combined dietary lutein and zeaxanthin, and this level of intake was correlated with a 43% lower risk for AMD. Additionally, this status was most readily achieved in subjects who frequently consumed dark green leafy vegetables such as spinach or collard greens. Unfortunately, studies conducted nearly a decade later have revealed that awareness among the general population is low in comparison to other eye diseases (e.g., glaucoma, cataracts) and chronic diseases (e.g., coronary heart disease, high blood pressure) (Rosenthal et al., 2003; Prahlad, 2002).

In 1984, Snodderly et al. first described the localization and distribution of the yellow macular pigment within the Henle fiber layer of the fovea (Snodderly et al. 1984a & 1984b). Shortly thereafter, lutein and zeaxanthin, two dietary xanthophylls, were recognized as the chemical compounds that comprise the macular pigment (Bone et al., 1985; Handelman et al., 1988). Xanthophylls refer to the class of oxygenated carotenoids found mostly in green plants. It has since been determined that the macula consists of 3 major carotenoids (Bone et al., 1993; Khachik et al., 2002). The third major pigment present was identified as (3R,3'S, *meso*)-zeaxanthin (referred to hereafter as *meso*-zeaxanthin). *meso*-Zeaxanthin is a stereoisomer of zeaxanthin that is of non-dietary origin (Bone et al., 1993; Khachik et al., 2002). These colored carotenoids in the macular region give the fovea its characteristic yellow color (hence the name macula lutea). In addition to lutein, zeaxanthin, and *meso*-zeaxanthin, a wide range of carotenoids have also been identified in human retinal pigment epithelium (RPE/choroid), ciliary body, iris, lens, and in the uveal tract and other tissues of the human eye (Khachik et al., 1997a; Bernstein et al., 2001; Khachik et al., 2002). Unless otherwise specified in this proposal, lutein and zeaxanthin refer to dietary (3R,3'R,6'R)-lutein and (3R,3'R)-zeaxanthin, respectively.

Lutein and zeaxanthin have been quantified in a wide range of fruits and vegetables (Humphries et al., 2003; USDA, 2004). Humphries et al. have shown that the best dietary sources of lutein and zeaxanthin are green leafy vegetables, yellow-orange fruits and vegetables, and to a lesser extent, foods containing egg yolk such as wheat and pasta products. It was observed that green vegetables tend to have higher ratios of lutein to

zeaxanthin, while the yellow-orange fruits and vegetables tends to have a lower lutein to zeaxanthin ratio (Humphries et al., 2003).

Prior to 1992, many studies routinely analyzed carotenoids in human blood and the HPLC techniques employed allowed for the separation and identification of only about seven carotenoids. In 1992, Khachik et al. used a combination of C_{18} reversed-phase and silica-based nitrile-bonded high performance liquid chromatography (HPLC) columns to effectively separate, identify, and quantify 25 carotenoids and 9 metabolites, and thus provided a detailed method for analysis of human plasma (Khachik et al., 1992a & 1992b). Later, the same researchers identified these carotenoids and their metabolites in human breast milk (Khachik et al., 1997b). The carotenoid metabolites identified in human serum and breast milk were not of dietary origin. In 1997, Khachik et al. reported that among 25 dietary carotenoids and 9 metabolites routinely found in human serum, lutein, zeaxanthin, and their metabolites were also present in human and monkey retinas (Khachik et al. 1997a). An extension of this work identified the complete spectrum of carotenoids in other ocular tissues of the human eye (RPE/choroid, ciliary body, iris, lens) (Bernstein et al., 2001). While lutein, zeaxanthin, and their metabolites were the major carotenoids found in human ocular tissues, lycopene and a wide range of other dietary carotenoids were detected in high concentrations in ciliary body and RPE/choroid.

Schalch reviewed a number of publications from the early 1990s and concluded that the cumulative data from various observational studies provided circumstantial evidence for the protective role of lutein and zeaxanthin in the retina against AMD (Schalch, 1992). Thereafter, Snodderly suggested potential mechanisms for the protective role of these

carotenoids in the human ocular tissues (Snodderly, 1995). These investigators suggested that lutein and zeaxanthin play a protective role in the macula in the prevention of AMD by absorbing short-wavelength visible light and thus preventing photochemical damage to cones and photoreceptors (Schalch, 1992; Snodderly et al., 1984a & 1984b).

Epidemiological and observational studies have revealed that low macular pigment optical density (MPOD) is associated with an increased risk of AMD (EDCCS, 1993; Beatty, 2001). MPOD refers to the concentration of carotenoids found in the macular region of the retina.

Since the early 1990s a main area of focus has been oxidative damage to photoreceptors that results from exposure to blue light. Two mechanisms have been proposed for the protective role of lutein and zeaxanthin; these are: (1) acting as optical filters by absorbing damaging short wavelength (blue) light; and (2) the antioxidant function by which these carotenoids quench oxygen free radicals and singlet oxygen that result from the simultaneous presence of light and oxygen in the macula (Kirschfield, 1982; Handelman, 1988; Snodderly, 1995; Landrum et al., 1997a; Beatty, 1999; Schalch, 1999).

In 1997, for the first time, Khachik et al. provided preliminary evidence for the photo-protective role of lutein and zeaxanthin in the retina by an antioxidant mechanism of action (Khachik et al., 1997a). This was accomplished by isolation, identification, and structural elucidation of lutein, zeaxanthin, and their oxidation products in the retinas of 11 human donor eyes and that of one monkey. While lutein, zeaxanthin, and a direct oxidation product of lutein were found to be the major carotenoids in the macula, 11 minor carotenoids were also identified. Based on these findings, Khachik et al. postulated a series

of oxidation-reduction reactions by which dietary lutein and zeaxanthin can be converted to their oxidation products in order to protect the macula against bright light and prevent AMD (Khachik et al., 1997a). As mentioned earlier, these investigators identified the metabolites of lutein and zeaxanthin in all ocular tissues of the human eye in their subsequent follow-up studies (Bernstein et al., 2001; Khachik et al., 2002). The proposed metabolic pathways of lutein and zeaxanthin in the human ocular tissues will be discussed later in this thesis. The epidemiological and supplementation studies presented are also described in detail later in this thesis.

The age-related eye disease study (AREDS) was one of the first large-scale human supplementation studies that investigated the protective role of carotenoids against AMD. This multi-center clinical intervention trial that began in 1992 and involved 3,640 subjects investigated the effects of supplementation with various combinations of antioxidant vitamins (including b-carotene) and zinc on the development and progression of cataracts and AMD (AREDS, 2000). Lutein and zeaxanthin, however, were not commercially available at the outset of planning this study and were therefore not included in the supplements. The results of this study showed a 25% decrease in the risk of AMD with the daily supplementation of 500 mg vitamin C, 400 IU vitamin E, 15 mg b-carotene, and 80 mg zinc (NEI, 2004).

The first human supplementation with lutein was conducted by Khachik et al. in which three healthy Caucasian males (non-smokers) between the ages of 42–59 were given oral supplements containing 10 mg/day of lutein dispersed in olive oil for 18 days (Khachik et al., 1995). Plasma carotenoid profiles of the subjects were monitored by HPLC at specific

intervals. The blood levels of lutein in all 3 subjects increased by 4- to 5-fold after one week of supplementation. In one subject lutein blood level increased from about 16 μg/dL (0.28 X 10^{-6} mol/L) to about 64 μg/dL (1.12 X 10^{-6} mol/L) resulting in the maximum absorption of this compound after one week of supplementation. The levels of the lutein oxidation products (metabolites) during this study increased significantly, confirming that the *in vivo* oxidation of lutein is indeed one of the key reactions in the metabolism of this carotenoid in humans. No apparent toxicity or side effects as a result of lutein ingestion was observed in the subjects.

In a similarly designed study by Khachik et al., one subject ingested 20 mg/day of lutein dispersed in olive oil for 21 days and the plasma carotenoid profile of the subject was monitored at various intervals up to 40 days (Khachik et al., 1997c). The blood levels of lutein increased by 9-fold from 0.21 μmol/L to 1.89 μmol/L within 3 weeks of treatment. At the end of the supplementation period, the levels of the lutein oxidative metabolites increased by 2- to 3-fold. A complete eye examination conducted after 21 days of supplementation revealed no unusual accumulation of lutein in the retina or ocular toxicity in the subject of the study.

The first supplementation study with zeaxanthin was also conducted by Khachik et al. in which 3 subjects ingested oral supplements containing 10 mg/day of zeaxanthin in olive oil for 3 weeks and the carotenoid plasma levels of the subjects were monitored at various intervals (Khachik et al., 1995). The blood levels of zeaxanthin in all three subjects increased by 4-fold after one week of supplementation. In addition, the plasma

concentrations of the oxidation products of lutein and zeaxanthin (metabolites) increased significantly.

In 1997, Landrum et al. conducted a study involving 2 subjects that were supplemented with lutein esters equivalent to 30 mg of free lutein per day for 140 days (Landrum et al., 1997b). In this study, serum lutein levels of the subjects increased by 10-fold within 20 days and plateaued at 1761 nmol/L and remained at this level for the duration of the study. After 40 days, these researchers observed an increase in MPOD at an average rate of 1.13±0.12 milliabsorbance units/day.

A recent double-blind randomized clinical trial investigated the dose response of elderly subjects with and without AMD to lutein at 3 supplemental doses (Moura et al., 2004). Forty-five subjects aged 60 years or older were divided into 3 groups: (1) no diagnosis of AMD, (2) middle stage of AMD, or (3) end stage of AMD, and were randomly assigned to receive one of three oral doses of lutein containing 5% zeaxanthin for 6 months. Serum lutein levels of all subjects increased, and subjects on doses of 2.5, 5, 10 mg/day of lutein reached a serum plateau of 450, 490, and 810 nmol/L, respectively. This study revealed that serum concentration of lutein was dose dependent and that the presence or absence of AMD did not interfere with serum levels of lutein (Moura et al., 2004).

The Veterans LAST Study was a double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation that involved 90 subjects with atrophic AMD and a mean age of about 75 (Richer et al., 2004). The purpose of this study was to investigate the effect of lutein alone and lutein in combination with other carotenoids, antioxidants, vitamins, and minerals on MPOD and central vision outcome measures in atrophic AMD. Richer et al. showed that daily supplementation of human subjects with

lutein alone or in combination with other antioxidant vitamins for one year effectively increased MPOD and improved glare recovery, near visual acuity and significantly improved most measures of quality of vision.

Although lutein and zeaxanthin have been commercially available and widely used in the United States as a nutritional supplement for nearly a decade, the safety and efficacy of these carotenoids had not yet been established. Meanwhile, because of the implication of lutein and zeaxanthin in the prevention and treatment of AMD and other ocular diseases, multi-center clinical trials with these carotenoids are in the planning stage by the NEI. Prior to the study discussed in this thesis, metabolic and toxicity studies with lutein and zeaxanthin had not been explored. The study outlined in this thesis was undertaken to investigate the safety and efficacy of chronic supplementation with high doses of lutein or zeaxanthin. Additionally, supplementation with a 1:1 combination of lutein and zeaxanthin had not been conducted so this study also sought to examine the efficacy of such a dose as well as possible interaction between these carotenoids. Female rhesus macaques (*Macaca mulatta*) were selected as an appropriate animal model due to the invasive nature of the proposed studies and possible toxicity associated with the administration of lutein and zeaxanthin at pharmaceutical doses. As described earlier, the distribution of lutein, zeaxanthin, and their metabolites in the ocular tissues of primates has been shown to be similar to that of humans which make this animal a suitable model for metabolic and toxicity studies. Justification for the selection of the animal model will be described later in the thesis.

LITERATURE REVIEW

Distribution of Carotenoids in the Macular Pigment

In 1945, George Wald first identified the yellow pigment in the human macula as a carotenoid member of the xanthophyll family (Wald, 1945). Xanthophylls include oxygenated carotenoids that are derived from green plants (e.g., lutein). Since then, the relationship between dietary xanthophylls and the optical density of the yellow macular pigment was established in a study in which primates were fed a xanthophyll-deficient diet (Malinow et al., 1980). In this study, animals that were fed a xanthophyll-deficient diet showed a reduction in the optical density of yellow macular pigment and more frequently developed drusen-like bodies. Drusen, which are extracellular deposits that accumulate between the retinal pigment epithelium (RPE) and Bruch's membrane, have been cited as possible precursors of AMD. Bruch's membrane, together with the RPE serves as the blood-retinal barrier and both are therefore critical in maintaining the integrity of the retina (Mares-Perlman & Klein, 1999). The decrease in macular pigment optical density observed by Malinow et al. was related to the absence of the major xanthophyll component of greens, the carotenoid lutein. In 1985, the macular pigments were first identified by Bone et al. as lutein [(3R,3'R,6'R)-β e-carotene-3,3'-diol] and zeaxanthin [(3R,3'R)-β,β-carotene-3,3'-diol] by HPLC analysis of retinal extracts (Bone et al., 1985). In 1988, these pigments were detected not only in the human macula, but throughout the entire retina (Handleman et al., 1988).

In 1993, the complete identification and stereochemistry of the human macular pigment was accomplished and the composition was shown to consist of: lutein

[(3R,3'R,6'R)-βe-carotene-3,3'-diol)], zeaxanthin [(3R,3'R)-β,β-cartotene-3,3'-diol)], and (3R,3'S, *meso*)-zeaxanthin [(3R,3'S)-β,β-carotene-3,3'-diol] (Bone et al., 1993). The structures of these carotenoids are shown in Figure 1. Unless specified in this thesis, lutein, zeaxanthin, and *meso*-zeaxanthin refer to dietary lutein [(3R,3'R,6'R)-β,ε-carotene-3,3'-diol), dietary zeaxanthin [3R,3'R)-β,β-carotene-3,3'-diol)], and (3R,3'S, *meso*)-zeaxanthin [(3R,3'S)-β,β-carotene-3,3'-diol].

Bone et al. also found that the ratio of lutein to zeaxanthin in donor retinas varied from the center of the macula outward. In the periphery of the retina (8.7–12.2 mm), the lutein to zeaxanthin ratio was found to be 2:1, and in the central region (0–.25 mm), the ratio was 1:2.4 with zeaxanthin predominating (Bone et al., 1988 & 1992). Additionally, the overall retinal carotenoid content was shown by these investigators to decrease considerably with increased eccentricity from the fovea. Further evidence to support the distribution of these carotenoids in the macula came from a study of postmortem human eyes (van Kuijk et al., 1997). In this study, lutein was found to be the predominant carotenoid associated with the rod photoreceptors, which are primarily located in the periphery of the retina.

In human serum, the ratio of lutein to zeaxanthin is considerably higher than in the retina, perhaps because of the comparable abundance of dietary lutein in most diets. A compelling finding was that approximately half of the zeaxanthin in the macula was dietary (3R,3'R)-zeaxanthin, and the remainder was *meso*-zeaxanthin, which is not of dietary origin (Bone et al., 1993; Khachik et al., 2002). *meso*-Zeaxanthin was found to account for less than 1% of the total zeaxanthin found in human blood. More recently, in a study of the *in vivo* transformations of dietary lutein and zeaxanthin, there was no

detectable concentration of *meso*-zeaxanthin found in the human plasma or liver tissues (Khachik et al., 2002). Unfortunately, there was no dietary history or plasma carotenoid profile available for the subject whose liver was analyzed, but high levels of dietary lutein and zeaxanthin were detected in this liver sample. The highest concentrations of *meso*-zeaxanthin relative to dietary zeaxanthin were found in the macula and RPE/choroid in the ocular tissues that were analyzed. This finding confirmed the *in vivo* conversion of dietary lutein to *meso*-zeaxanthin in the human ocular tissues as described by Khachik et al. in an earlier publication (Khachik et al., 1997a).

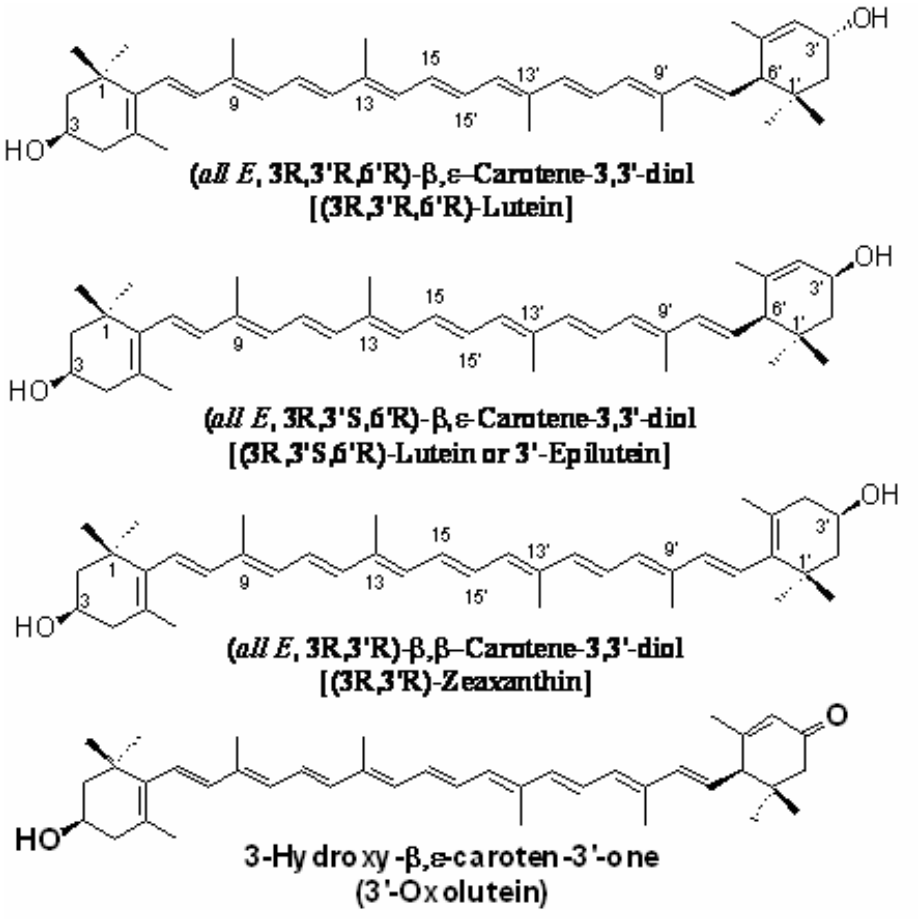


Figure 1. Chemical structures of lutein, zeaxanthin, and their metabolites.

Dietary Sources of Lutein and Zeaxanthin

Lutein and zeaxanthin are found in a variety of foods commonly consumed in Western diets. The best sources of lutein and zeaxanthin are green leafy vegetables, yellow-orange fruits and vegetables, and to a lesser extent, wheat and pasta products; the best sources of β-carotene and lycopene are carrots and tomatoes, respectively (Mangels et al., 1993). In 2003, a study was published that, for the first time quantified (3R,3'R,6'R)-lutein, (3R,3'R)-zeaxanthin, and their (*E/Z*)-geometrical isomers in a variety of fruits, vegetables, wheat, and pasta products (Humphries et al., 2003). Prior to this, most data that quantified lutein and zeaxanthin in foods were presented as the combined concentration of these carotenoids plus their geometrical isomers due to the difficulties associated with separation of these carotenoids by high performance liquid chromatography (HPLC). An extensive listing of the commonly consumed foods in the United States and their combined lutein and zeaxanthin content is available at <http://www.nal.usda.gov/fnic/foodcomp/Data/SR17/wtrank/sr17w338.pdf> (USDA, 2004).

The 2003 study by Humphries et al. revealed nearly identical qualitative HPLC profiles among green leafy vegetables (with the exception of romaine lettuce), and among these, the highest concentration of lutein and zeaxanthin was found in kale, parsley, spinach, and collards.

Additionally, in all of the green fruits and vegetables analyzed, the ratio of lutein to zeaxanthin varied from 12 to 63 (Humphries et al., 2003). Romaine lettuce was the exception due to the presence of significant quantities of the rare dihydroxycarotenoid lactucaxanthin. Other sources of lutein and zeaxanthin include green beans, lima beans,

broccoli, lettuce, and peas. Yellow-orange fruits and vegetables including butternut squash, corn, oranges, and nectarines were identified as good sources of zeaxanthin. This study found that the ratio of lutein to zeaxanthin was close to one in these foods; this is with the exception of corn and nectarines which contained higher concentrations of zeaxanthin than lutein. Of the wheat and pasta products analyzed, the levels of lutein and zeaxanthin were lower than that of the yellow-orange and green fruits and vegetables. The presence of these carotenoids in wheat and pasta products was attributed to the presence of egg yolk, which contains moderate levels of dietary lutein and zeaxanthin. This study enabled the identification of good sources of lutein and zeaxanthin.

Epidemiological studies have shown that a decreased risk of neovascular AMD is associated with high plasma concentrations of carotenoids (lutein, zeaxanthin, β-carotene, α-carotene, β-cryptoxanthin, α-cryptoxanthin, and lycopene) (EDCCS, 1992; Gale et al., 2003). While serum carotenoid levels increase quickly with supplementation (Khachik et al., 1995; Landrum et al., 1997a), it has been shown that the increase in MPOD as a result of supplementation or dietary intake of these carotenoids is a much slower process (Landrum et al., 1997b; Toyoda et al., 2002). Although the exact mechanism for uptake of dietary lutein and zeaxanthin into the macula has not yet been elucidated, retinal tubulin, a carotenoid binding protein has been suggested as a mediator for uptake of these carotenoids in the fovea (Bernstein et al., 1997). More recently, a pi isoform of glutathione *s*-transferase (GSTP1) has been identified as a xanthophyll-binding protein in the human macula that displays a strong affinity for dietary zeaxanthin and *meso*-zeaxanthin and, and to a lesser extent, lutein (Prakash et al., 2004).

Proposed Metabolic Pathways of Lutein and Zeaxanthin in Humans

It has been hypothesized that lutein and zeaxanthin in the human macula provide protection against AMD by two mechanisms. First, lutein and zeaxanthin absorb short-wavelength blue light between 400–475 nm, which peaks at 440 nm, and has the most damaging effects on the retina. The main absorption maxima of lutein and zeaxanthin in organic solvents are 448 nm and 454 nm, respectively (Britton, 1995). It has been suggested that lutein and zeaxanthin in the macula act as optical filters by absorbing damaging blue light, and as a consequence, reduce and/or prevent the amount of light-induced oxidative damage. Secondly, carotenoids are potent antioxidants and can quench reactive oxygen species in the macula and thereby prevent damage to the cones and photoreceptors (Schalch et al., 1999; Beatty et al., 1999; Liebler et al., 1997). The retina is highly susceptible to oxidative damage due to the high rate of metabolic activity and the simultaneous presence of high levels of oxygen and light.

The initial hypothesis of this protective effect was based on the previously observed concentrations of lutein and zeaxanthin in the macula, epidemiological data, and animal studies (EDCCS, 1992; EDCCS, 1993; Seddon et al., 1994; Malinow et al., 1980; Kirschfeld, 1982; Krinsky, 1989; Snodderly, 1995). A cumulative body of data was reviewed in 1992 by Schalch, who concluded that the presence of lutein and zeaxanthin in human and monkey retinas does indeed serve a specific purpose (Schalch, 1992). It was noted that of the 10 dietary carotenoids found in human blood, lutein and zeaxanthin are present in the macula due to their excellent ability to quench oxygen free radicals and singlet oxygen, both of which are generated in this region due to the concurrent presence of light and oxygen (Schalch, 1999). Additionally, Landrum et al. provided evidence for

the possible role of these carotenoids by showing the loss of these macular pigments throughout the macula in AMD donor eyes in comparison with control eyes (Landrum et al., 1997). It is now believed that the presence of these carotenoids in the macula may retard some of the destructive processes that occur in the retina and RPE that may lead to AMD. For a complete review of the evidence supporting the protective role of lutein and zeaxanthin against AMD see the publications of by Snodderly, 1995; Schalch et al., 1999; and Landrum & Bone, 2001.

The discovery of the metabolites and several oxidation products of lutein and zeaxanthin that are not of dietary origin in the human retina led to a postulated metabolic transformation by which dietary lutein and zeaxanthin in the retina may be converted to their metabolites (Khachik et al., 1995; Bernstein et al., 2001; Khachik et al., 2002). The presence of the oxidation products of lutein and zeaxanthin in the human retina provided preliminary evidence for the protective role of these carotenoids as antioxidants (Khachik et al., 1995; Khachik et al., 1997a; Landrum & Bone, 2001). According to the metabolic transformations proposed by Khachik et al., (3'R,3'S, *meso*)-zeaxanthin, (3R,3'R,6'R)-lutein, and (3R,3'R)-zeaxanthin may be interconverted by a series of oxidation-reduction and double-bond isomerization reactions as shown in Figure 2. The 3 types of reactions that may take place are: 1) the oxidation of the allylic hydroxyl group of the ϵ-end group of lutein to give an α,β-unsaturated ketocarotenoid (e.g., 3'-oxolutein), 2) the reduction of the resulting ketocarotenoid via epimerization at C-3' to form 3'-epilutein, and 3) stereospecific double bond isomerization of the β-end group of dietary zeaxanthin to form 3'-epilutein, and by a similar mechanism, the conversion of dietary lutein to (3R,3'R, *meso*)-zeaxanthin. The carotenoids shown in Figure 2 have all been detected in

human plasma and/or ocular tissues (Khachik et al., 1992a; Khachik et al., 1992b; Khachik et al., 1997a; Khachik et al., 1997c; Khachik et al., 1998; Bernstein et al., 2001). While the sources of 3'-oxolutein and 3'-epilutein in the retina may be due to the presence of these carotenoids in circulating blood, this is an unlikely scenario for *meso*-zeaxanthin. This is because it has now been established that *meso*-zeaxanthin is absent in human plasma and liver but present in nearly all of the ocular tissues (Khachik et al., 2002).

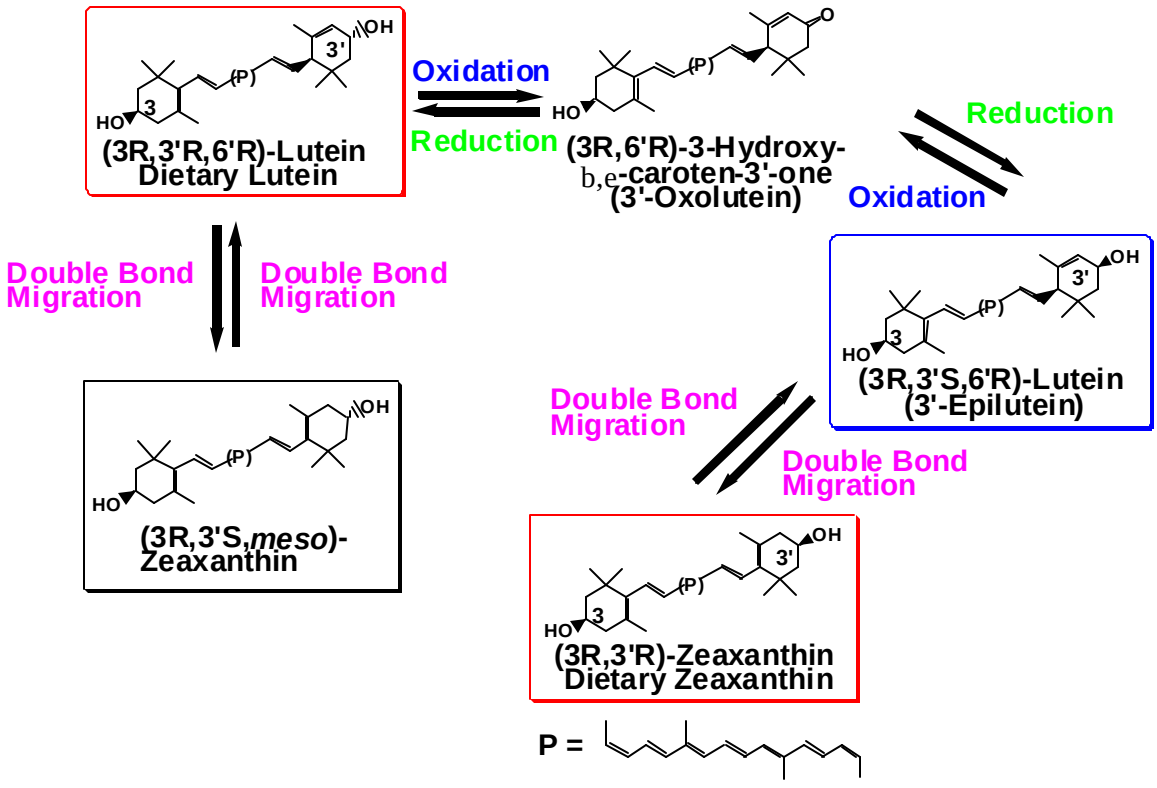


Figure 2. Proposed metabolic pathways of dietary lutein and zeaxanthin in humans.

Epidemiological Studies and Risk Factors Associated with AMD

Epidemiological studies have provided insight into the occurrence of the 2 forms of AMD: wet and dry AMD. AMD is defined as the late stage of age-related maculopathy (ARM), and exists as geographic atrophy (the end stage of dry AMD), and choroidal neovascularization (wet AMD). ARM is most common in persons older than 50 years of age, and the presence of soft drusen (≥63 μm), choroidal hyperpigmentation associated with drusen, and depigmentation of the RPE are the characteristic symptoms. The extracellular deposits know as drusen accumulate between the RPE and Bruch's membrane and vary in size and morphology (Mares-Perlman & Klein, 1999). It was recently shown that photoreceptors of both the macular and extramacular region overlying drusen deposits exhibit both structural and molecular abnormalities (Johnson et al., 2003).

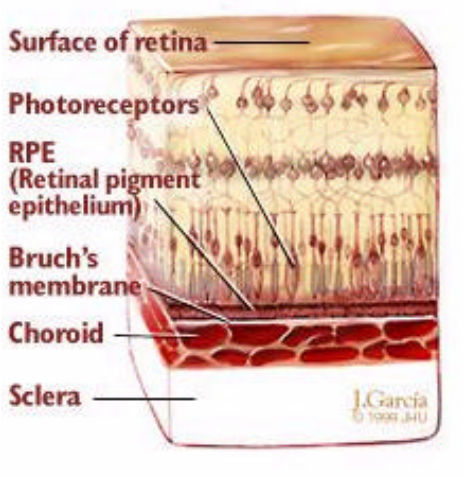


Figure 3. Anatomy of retina and posterior eye. Source: < http://www.hopkinsmedicine.org >.

Prevalence studies of AMD have been conducted mainly in industrialized countries. Three large population-based epidemiological studies have provided estimates for the prevalence of geographic atrophy and neovascular AMD. They are: the Beaver Dam Study in Wisconsin (Klein et al, 1992); the Rotterdam Study in The Netherlands (Vingerling et al., 1995); and the Blue Mountains Eye Study in Australia (Mitchell et al., 1995). The data from these studies revealed the following figures for the prevalence of geographic atrophy: 0.44% (the Beaver Dam Study), 0.66% (the Rotterdam Study), and 0.45% (the Blue Mountains Eye Study). Meanwhile, the same studies reported that the prevalence of neovascular AMD was: 0.88%, 0.72%, and 1.20%, respectively.

AMD is a multifactorial disorder. While the etiology of AMD is unknown, it has been linked to the combination of numerous risk factors. One of the major risk factors for AMD is age. The Framingham Eye Study showed that 28% of individuals between the ages of 75 and 85 had AMD compared to 22% of individuals between the ages of 52 and 74 (Kahn et al., 1977). Additionally, the Beaver Dam Eye Study, which investigated the 10-year incidence of ARM showed a significant increase in the incidence of this disease with age. This study revealed a 19.5% increase of retinal pigment abnormality in subjects aged 75 or older at baseline from the outset of the study to its conclusion compared with a 0.8% increase in individuals aged 43 to 54 at baseline (Klein et al., 2002).

Family history has also been shown to be an important risk factor associated with late AMD and early ARM (Smith et al., 1998). Several studies with twins proposed genetic influence as an AMD risk factor, although the relative importance of genetic versus environmental factors was not defined (Meyers & Zachary, 1988; Klein et al., 1994). In a study that compared monozygotic twins to dizygotic twins in order to exclude

the influence of shared family environment, results showed 45% heritability at the early stage of ARM (Hammond et al., 2002).

More recently, variants of several genes have been identified as possible risk factors for AMD. A single-nucleotide polymorphism (SNP) in the complement factor H (CFH/HF1) gene has been associated with an increased risk of AMD (Klein et al., 2005; Hageman et al., 2005). CFH plays a critical regulatory role in the complement system of innate immunity which protects against infection and attacks diseased cells. It has been hypothesized that inappropriate complement activation which causes an abnormal inflammatory response may result in AMD by way of tissue damage and cell death. The haplotype N1 within the CFH gene increases the risk for AMD significantly. In a whole-genome case-control association study that used a subset of participants from the Age-Related Eye Disease Study (AREDS) (96 case and 50 control subjects), being heterozygous for this haplotype increased the risk for AMD by a factor of 4.6 and being homozygous for this haplotype increased the risk for AMD by a factor of 7.4 (Klein et al, 2005).

In a study of 2 independent cohorts comprised of 900 AMD cases and 400 matched controls that analyzed genetic variations in HF1, multiple HF1 variants were associated with elevated or reduced risk of AMD (Hageman et al., 2005). Strong associations with AMD were found among 3 SNPs for this gene; the strongest was the A473A variant in exon 10 (rs2274700) (odds ratio = 3.42, 95% CI). Additionally, 2 common protective haplotypes were identified in 34% of controls and 18% of cases. Results suggest that the HF1 protein associated with the at-risk HF1 haplotype(s) may attenuate complement inhibitory function leading to excessive amounts of membrane

attack complex and consequent tissue damage. This evidence supports the hypothesis that a specific and common haplotype of HF1, a complement regulator, predisposes individuals to AMD (Hageman et al., 2005; Klein et al, 2005).

Also implicated as a risk factor for AMD are variants of the ABCA4 gene. In a study that screened over 1,000 unrelated AMD patients, the 2 most frequent AMD-associated variants in ABCR were investigated (Allikmets et al., 2000). The risk of AMD was 3-fold higher for the variant D2177N and 5-fold higher for the variant G1961E.

The ApoE gene has also been implicated as a risk factor for AMD (Schmidt et al., 2000; Malek et al., 2005). In a recent experimental study, a new animal model that was developed, the apoE TR mouse, manifests a comprehensive range of human AMD-like pathologies (Malek et al., 2005). The homozygous ApoE4 genotype when combined with advanced age and high fat cholesterol-rich diets produced neovascularization (NV) associated with advanced AMD among approximately 18% of the animals in this group and was not observed in the age and diet matched group that expressed the other two apoE alleles. Additionally, the proteins detected in the NV lesions of these mice were consistent with those seen in human NV.

Being Caucasian appears to increase the risk of AMD. The white population has been shown to have a higher prevalence of ARM (5.6%) when compared to that of the black population (3.7%) (Klein et al.,1999). The Baltimore Eye Survey which involved a total of 5,431 participants found that the prevalence of AMD among whites over 70 years of age was 2.1%, while there were no cases of AMD detected among the 243 black participants in this age group (Friedman et al., 1999).

Gender has also been shown to be a risk factor for AMD. The Beaver Dam Eye Study found that the prevalence of exudative AMD was 6.7% for women and 2.6% for men among the Caucasian population in the study (Klein et al., 1992). The gender difference was also apparent among the African American population as women were twice as likely to have AMD as men (Pieramici et al., 1994). An interesting point to note is that MPOD has been shown to be lower in women; men were shown to have an average of 38% or higher MPOD than women (Hammond et al., 1996). Low MPOD causes increased exposure to damaging blue light which may explain the higher prevalence of AMD seen in women.

Cigarette smoking is an important modifiable risk factor for AMD. Smoking has been associated to a greater extent with neovascular AMD rather than with geographic atrophy (Hyman et al., 1992). The Eye-Disease Case Control Study Group also found a strong association between risk of neovascular AMD and current smokers (EDCCS, 1992). Additonally, the Blue Mountains Eye Study assessed the relationship between baseline smoking and the 5-year incidence of late and early ARM in 3,654 subjects aged 49 years or older (Blue Mountains Eye Study, 2002). This study found that current smokers had an increased risk in pigment abnormalities and of developed late ARM at a significantly earlier age than former smokers and those who had never been smokers. Additionally, the POLA Study found former smokers to remain at increased risk for AMD (Delcourt et al., 1998).

While epidemiological studies have investigated the association between AMD and cardiovascular disease and related risk factors such as high blood pressure and high serum cholesterol, the relationship is still unclear. The Eye Case Control Study Group

(EDCCS, 1992), did not find a significant association between hypertension and neovascular AMD, but did find a significant trend with higher systolic blood pressure. Conversely, the AMD Risk Factors Study Group found a positive association between neovascular AMD and diastolic blood pressure greater than 95 mm Hg (odds ratio [OR] = 4.4) (Hyman et al., 2000). Additionally, a direct association between elevated HDL cholesterol levels and the incidence of AMD has been suggested but the reason is not known (Hyman et al., 2000; Klein et al., 2003a). Several epidemiological studies did not find any association between AMD and hypertension, stroke, angina, and acute myocardial infarction (Smith et al., 1998; Klein et al., 2003b). Examined cumulatively, the results of studies investigating these associations are inconclusive.

Chronic or abnormal inflammatory responses have emerged as possible indicators of AMD risk. It has been noted that cardiovascular disease and AMD share common antecedents and biomarkers of systemic inflammation such as elevated C-Reactive Protein (CRP) levels (Snow et al., 1999). In a study of over 250 subjects with a mean age of 72 years, analysis of biomarkers of inflammation showed: 1) physical activity was inversely related to CRP, interleukin-6 (IL-6), and tumor necrosis factor-a-R2 (TNF-a-R2); 2) smoking was associated with all biomarkers but vascular cell adhesion molecule-1 (VCAM-1); and CVD; 3) systolic blood pressure were positively related to most markers; and 4) body mass was positively associated with CRP, TNF-a-R2, and VCAM-1. Statistical analysis from this study showed a 2-fold greater risk of progression of AMD among the highest quartile of CRP, and odds ratio of 1.81 for the highest quartile of IL-6; and both smoking and BMI were positively related high levels of CRP and IL-6 and to AMD. The identification of the factor H gene (HF1) as an AMD risk gene provides

support for the role of inflammation in AMD (Bok et al., 2005; Klein et al., 2005). HF1 codes for a protein involved in the innate system, the body's first line of defense against infection. A broader haplotype of the regulator of complement activation (RCA) gene located nearby on the same chromosome was recently found to be present in nearly half of those with AMD compared to approximately 29 % of controls (Hageman et al., 2005). It has been hypothesized that dysfunction of the complement system which leads to chronic inflammation could contribute to chronic diseases and, in the case of AMD, disruption of Bruch's membrane and subsequent lesion formation (Hageman et al., 2005). Prolonged inflammatory response, a condition seen in obesity and in other chronic diseases, may provide a link between the aforementioned diseases and increased AMD risk.

Obesity, high BMI, and waist-to-hip ratio have been cited as risk factors for AMD (AREDS 2000). In a study of 680 men and women from two sites in the United States, there was an inverse relationship between MPOD and BMI ($p < 0.0008$) and MPOD and body fat percentage ($p < 0.01$) (B.R. Hammond et al., 2002). These relationships were observed only in the group of subjects with a BMI above 29 and fat percentage above 27%. However, dietary intake of lutein and zeaxanthin was also lower in these groups. In the 5-year follow-up of the Beaver Dam Eye Study (n=3722), researchers found a significant association between age-related maculopathy and both BMI and waist-to-hip ratio among women, but found that waist-to-hip ratio was more strongly associated with nearly every outcome (Klein et al., 2001). These researchers found little difference between BMI and waist-to-hip ratio as indicators of age-related eye disease among men

(Klein et al., 2001). While there is a greater range of waist-to-hip ratios in women than men, these results suggest the distribution of adiposity might be related to AMD risk.

There is some evidence to suggest that light iris color (i.e., blue, green irises) is associated with increased risk of AMD, but this is controversial. Analysis of the cross-sectional data from the Blue Mountain Study revealed a significant association between blue iris color and an increased risk of early ARM (odds ratio=1.45) and late ARM (odds ratio=1.69) (Mitchell et al., 1998). However, the Beaver Dam Eye Study showed that individuals with brown eyes were more likely to develop soft indistinct drusen than those with blue irises (Tomany et al., 2003). Additionally, the Eye Disease Case Control Study group found no significant association between iris color and AMD (EDCCS, 1992).

Dietary factors have been cited as possible factors in the development of AMD. Dietary fat is one potential risk factor for AMD, however results from studies have been inconsistent. The Beaver Dam Eye Study reported that subjects in the highest quintile of saturated fatty acid intake were at a significantly higher risk for early AMD than those in the lowest quintile. (Mares-Perlman et al.,1995a). Alternatively, The Blue Mountains Study did not find a significant association between AMD and saturated fat, but instead found a relationship between intake of monounsaturated fat and a significant borderline increase in the risk of early AMD (Smith et al., 2000). In yet another study, polyunsaturated fat, such as linoleic acid, was associated with risk of AMD (Seddon et al., 2001).

Antioxidants, alternatively, may prevent the progression of AMD. Carotenoids have been cited as being among the most effective and abundant dietary antioxidants that

prevent oxidative damage by quenching singlet oxygen and other reactive oxygen species in liposomes, lipoproteins, membranes, and cells (Krinsky, 1989).

Evidence for the protective role of carotenoids against AMD was collected in a study conducted by the Eye Disease Case-Control Study Group, specifically for the beneficial role of carotenoids in the prevention of neovascular (wet) AMD (EDCCS, 1993). The study subjects included 421 patients with neovascular AMD and 615 controls, and the goal was to evaluate antioxidant status (including vitamins C and E, carotenoids, and selenium) and the risk factors for wet AMD. The results of this study showed that the subjects with medium and high serum carotenoid levels as compared to those with low serum carotenoid concentrations were at significantly reduced risk of neovascular AMD. However, a nested case-control study (Beaver Dam Eye Study), examined subjects with retinal pigment abnormalities with the presence of drusen (n = 127), late AMD/geographic atrophy (n = 9), or exudative AMD (n = 31), and an equal number of controls (n = 167), and found no correlation between serum lutein and zeaxanthin concentrations and risk of AMD. This study revealed that the subjects with serum lycopene levels in the lowest quintile were twice as likely to develop AMD (Mares-Perlman et al., 1995b).

Another study, published in 1994 reported that diets high in fruits and vegetables, specifically those rich in lutein and zeaxanthin, were correlated with a reduced risk of AMD (Seddon et al., 1994). Seddon et al. examined 356 case subjects with advanced stage of AMD and 520 control subjects who were within the same age range and from the same geographic region, but had ocular diseases other than AMD. This study showed that the subjects in the highest quintile of carotenoid intake had a 43% lower risk for AMD compared with those in the lowest quintile. More specifically, frequent consumption of

spinach or collard greens, which contain high concentrations of lutein and zeaxanthin, was associated with a substantially lower risk for AMD.

The epidemiological and observational studies that provided such promising results in the early 1990s were the impetus for the large-scale clinical intervention trial with β-carotene known as the Age-Related Eye Disease Study (AREDS, 2000; Sackett & Schenning, 2002). AREDS began in 1992 and was concluded in 2001, and involved over 4,600 subjects at 11 eye disease centers across the United States. The intent of this study was to examine whether long-term supplementation of patients at various stages of AMD or cataract with β-carotene, vitamins C and E, and zinc, alone or in combination, could prevent or slow the progression of these eye diseases. An important point to note is that at the outset of planning AREDS, lutein and zeaxanthin were not yet commercially available, but β-carotene was both commercially available and accepted as a safe dietary supplement. Although β-carotene does not accumulate in the retina, it was selected for this study based on its role in the human visual cycle as a precursor of retinol (vitamin A). The subjects of this study were categorized into one of 4 groups: 1) no diagnosis of AMD, 2) early stage of AMD, 3) intermediate AMD, and 4) advanced stage of AMD. Nutritional supplements given to members of each group were randomized. Results of this study showed that a daily dose of 500 mg vitamin C, 400 I.U. vitamin E, 15 mg β-carotene, and 80 mg zinc oxide with 2 mg cupric oxide reduced the risk of developing advanced AMD by about 25% in individuals at high risk for developing advanced AMD (i.e., those with intermediate AMD or advanced AMD in one eye) (AREDS, 2000; Sacket & Schenning, 2002).

Because lutein and zeaxanthin are concentrated in the human retina and it has been suggested that they have possible protective roles against disease, blood levels of these carotenoids were examined in 7059 participants of the Third National Health and Nutrition Examination Survey aged 40 and over (Gruber et al., 2004). It was shown that lower serum lutein and zeaxanthin were significantly associated with smoking, heavy drinking, higher fat-free mass, being white, female, or not being physically active, having lower dietary cholesterol, and the presence of inflammatory markers such as higher white blood cell count, and high levels of C-reactive protein ($p < 0.05$). This study, in finding associations between low serum concentrations of lutein and zeaxanthin and previously identified risk factors for AMD further demonstrates the multi-factorial nature of this disease. Unfortunately, the results provide no information about the relative contribution of the individual risk factors to AMD risk.

Supplementation Studies with Lutein and Zeaxanthin

There have been few studies involving human supplementation of lutein and/or zeaxanthin; these studies provide a foundation for current research. Results of the first human studies of supplementation with lutein and zeaxanthin were published in 1995 (Khachik et al., 1995). In these studies, Khachik and 2 colleagues were the subjects of a lutein supplementation study. All 3 subjects were healthy nonsmoking Caucasian males between the ages of 42 and 59. Subjects were placed on a restricted diet that excluded green and yellow-orange fruits and vegetables containing lutein, and they were supplemented daily with 10 mg oral doses of lutein that were dispersed in olive oil. Otherwise, subjects were on self-selecting diets and kept dietary records throughout the

study. Baseline levels of plasma carotenoids were determined 25 and 11 days prior to the supplementation period (3 data points: days -25, -11, and baseline). Plasma carotenoid profiles were monitored at baseline and on days 2, 4, 7, 18, 26, 33, 40, and 57, and analyzed by HPLC. Lutein serum levels in all 3 subjects increased by 4- to 5-fold after one week of supplementation and peaked in one subject after one week of supplementation. Additionally, the levels of lutein oxidation products during this study increased significantly, providing preliminary evidence for the *in vivo* oxidation of lutein as a result of the metabolism of this carotenoid (Khachik et al., 1995).

A similar study was conducted by the same researchers in order to investigate the effects of short-term zeaxanthin supplementation on serum carotenoid levels (Khachik et al., 1995). The 3 subjects ingested 10 mg/day of zeaxanthin orally for 3 weeks. After one week of supplementation, the plasma zeaxanthin concentrations increased by 4-fold in all subjects, and plasma concentrations of lutein, 3'-epilutein, and the oxidation products (ketocarotenoids) of these carotenoids increased significantly.

Another study conducted by the same researchers sought to examine the short-term effects of a higher daily dose of lutein. In this study, one subject ingested a daily dose of 20 mg of lutein for a period of 21 days (Khachik et al., 1997c). The supplements were prepared in the same manner as previously described. Serum analysis was performed at baseline and on days 2, 4, 7, 9, 11, 15, 18, 18, 21, 25, 31, and 39 that measured the levels of 22 carotenoids, vitamin A, and vitamin E (α- and ?-tocopherol). There was a 9-fold increase in lutein serum levels of subjects from 12 μg/dL at baseline to 108 μg/dL after 3 weeks of supplementation. At the end of the supplementation period, the levels of lutein oxidation products were shown to increase by 2- to 3-fold. Because of

the known accumulation of lutein in the retinas, a complete eye examination of the subject was performed after 21 days of supplementation at the National Eye Institute. No unusual accumulation of lutein in the retina or ocular toxicity was observed.

The Veterans Lutein Antioxidant Supplementation Trail (LAST) investigated the effects of long-term lutein supplementation in patients with atrophic AMD (Richer et al., 2004). This double-masked, placebo-controlled, randomized trial of lutein and antioxidant supplementation involved 90 subjects with a mean age of about 75. Subjects were randomly assigned to one of the following 3 treatments for 12 months: 1) supplementation with 10 mg/day of lutein, 2) supplementation with 10 mg/day of lutein plus a wide range of antioxidants, vitamins, and minerals including zinc (L/A), and 3) administration of a placebo. Lutein in the supplements was in its non-esterified form to approximate food-equivalent lutein intake from spinach. The results of this study demonstrated that long-term supplementation (12 months) of lutein alone or in combination with additional carotenoids, antioxidants, vitamins, and minerals significantly improved MPOD, glare recovery, near visual acuity, and most measures of quality of vision. Additionally, in the patients receiving L/A supplementation, there was no observed progression of AMD retinopathy. However, while it was shown that improved visual function resulted from lutein supplementation, the researchers of this study identified the preliminary nature of the results due to limitations such as the small number of subjects and the population studied, as well as a lack of statistical significance among the 3 groups.

Another recent human supplementation study involving 45 subjects aged 60 or older investigated the association of 3 daily doses of orally ingested lutein supplements

with the serum levels of this carotenoid among 3 groups: 1) those without AMD, 2) those with middle stage AMD, and 3) those with end stage AMD (Moura et al., 2004; Chew et al., 2003). The supplemental doses of lutein, which contained 5% zeaxanthin, were 2.5, 5, and 10 mg/day. The data from this study showed a direct correlation between serum lutein concentrations and the 3 dose levels for all subjects, revealing that serum lutein concentration is dose-dependent. Serum concentrations of lutein increased in all subjects, and after 3 weeks of supplementation all groups reached a plateau. Subjects on doses of 2.5, 5, and 10 mg/day of lutein reached a serum plateau of 450, 490, and 810 nmol/L, respectively. Subjects given a dose of 10 mg/day showed a 3- to 4-fold increase in serum lutein levels after 3 weeks, while the subjects receiving 2.5 and 5 mg/day lutein doses showed almost a 2-fold increase. There was no significant difference between these 2 treatment groups (2.5 and 5 mg/day) until after week 25 of supplementation when subjects receiving 2.5 mg/day of lutein showed a decrease in serum plasma levels. It was shown that the presence or absence of AMD did not interfere with serum levels of lutein. Additionally, all subjects showed an increase in serum levels of 3'-oxolutein and 3'-epilutein (lutein and zeaxanthin metabolites), which supports the metabolic pathways previously discussed in this proposal (see Figure 2) (Khachik et al., 1995; Khachik et al., 1998; Khachik et al., 2002). Overall, the results from this study suggested that supplementation with a dose of at least 5 mg/day of lutein would provide the serum lutein level needed to lower the risk of AMD (Moura et al., 2004). This correlates with the plasma lutein level reported by the Eye Disease Case-Control Study to be associated with the lowest risk of AMD (670 nm/L) (EDCCS, 1993).

A series of recently published papers presented the results of a long-term study involving 18 Rhesus monkeys that were raised from birth until 7–16 years of age on a semipurified xanthophyll-free diet. Six of these primates were then fed pure lutein supplements (L-treated) and 6 with fed pure zeaxanthin supplements (Z-treated) at a daily dose of 2.2 mg/kg (3.9 µmol/kg) for 24–56 weeks; 6 were maintained on the xanthophyll-free diet until death (Neuringer et al., 2004; Leung et al., 2004; Johnson et al., 2005; Leung et al., 2005). These 3 groups were subdivided and assigned to diets containing either low or adequate n-3 fatty acids to see whether n-3 fatty acid status affects uptake of lutein or zeaxanthin into blood or tissues. Serum carotenoid levels and MPOD, which were measured at baseline and various intervals, were compared to control monkeys fed a stock diet (n=15) that provided a daily dose of ~150 µg (0.26 µmol)/kg of lutein and ~135 µg (0.26 µmol)/kg of zeaxanthin. Reversed-phase HPLC was used for qualitative and quantitative analysis of serum, and two-wavelength monochromatic fundus reflectometry and monochromatic fundus photographs were used to determine MPOD. After primate sacrifice the central retinas were serially sectioned and the number of RPE cells were counted and compared to the data from 15 animals fed the stock diet described (Leung et al., 2004).

Results showed that prior to supplementation, primates on the semipurified diet had no measurable serum lutein or zeaxanthin, while the stock diet-fed primates had a mean concentration of 0.074 ± 0.009 µmol/L of lutein (*trans* form only) and 0.081 ± 0.007 µmol/L of combined *cis* and *trans* zeaxanthin (Neuringer et al., 2004). After 2 weeks of supplementation the L-treated and Z-treated animals' serum xanthophylls levels exceeded those of the stock diet-fed animals and reached levels approximately 10-fold for

lutein and 10- to 20-fold for zeaxanthin compared to those of the control group. The researchers noted high inter-individual variability as the reason for the serum level differences between the L-treated and Z-treated primates not reaching statistical significance ($p = 0.12$) using repeated measures ANOVA. MPOD measured within the central 1mm of the retina of the xanthophyll-free primates were zero or very low. In the L-treated and Z-treated primates, MPODs increased during the first 24 to 32 weeks, but showed no consistent increases thereafter. No significant differences were observed between the L- and Z-treated animals. Both serum and ocular tissue data showed no effect of n-3 fatty acid status. It was also found that MPOD was not significantly related to total serum xanthophylls concentrations. While MPOD of the primates raised on a xanthophylls-free diet increased significantly, levels were only about half that of the primates fed stock diets.

Retina analysis revealed that foveal and parafoveal RPE cell densities increased with age and that the xanthophyll-free monkeys had a dip in the cell density profile of the RPE, unlike the control monkeys (Leung et al., 2004). After supplementation, n-3 fatty acid status seemed to interact with the supplemental lutein or zeaxanthin causing asymmetries in the RPE profile leading the researchers to conclude that adequate xanthophylls and n-3 fatty acids are essential for developing and maintaining normal distribution and/or maintenance of such a distribution.

A third paper published about this group of xanthophyll-free primates examined the effects of the aforementioned lutein or zeaxanthin supplementation on serum, adipose tissue, and retina concentrations of these carotenoids in the primates (Johnson et al., 2005). Results showed that adipose tissue, as well as the serum and retinas of the

xanthophyll-free primates had no lutein or zeaxanthin, but that concentrations increased significantly in both the L-treated and Z-treated groups after supplementation. Further, lutein and non-dietary *meso*-zeaxanthin were detected in the retina of the L-treated animals, but only all-*trans* zeaxanthin and not *meso*-zeaxanthin was detected in the Z-treated animals. The researchers concluded that lutein is a precursor of *meso*-zeaxanthin.

Another study of the xanthophyll-free primates examined the density of S-cone and rod cells in the foveal region (Leung et al., 2005). Serial sections 2 µm thick from the region containing the foveal depression were cut and outer segments of S-cones and nuclei of rods were counted. Results showed no consistent effect of n-3 fatty acid status or xanthophyll supplementation on the density profiles. There was, however, high inter-individual variability. Data suggested that some of the animals were resistant to the imposed nutritional manipulations while others may have been affected.

The study presented in this thesis followed a logical progression to the recent supplementation studies discussed in this review by investigating chronic high-dose supplementation with lutein or zeaxanthin in primates. While supplemental lutein at doses as high as 10 mg/day resulted in no interaction with other carotenoids, retinol, and α- and γ-tocopherols, and no adverse side effects, there were no data available for the effects of long-term supplementation with pharmaceutical doses of this carotenoid. Therefore, a study to begin investigating the effect of long-term supplementation with pharmaceutical doses of lutein was needed. Pharmaceutical doses refer to doses significantly higher than the average dietary intake levels. Also, there had been no studies to investigate the effects of long-term supplementation with pharmaceutical doses of zeaxanthin. This study also sought to investigate the effects of supplementation with a 1:1

combination of lutein and zeaxanthin on the plasma and tissue deposition of these carotenoids and their metabolites, which had not previously been investigated. Prior to the studies described in this thesis, the only supplementation study that has been conducted with zeaxanthin was a short-term study involving three subjects supplemented with a dose of 10 mg/day (Khachik et al., 1995).

Because of the invasive nature of the studies undertaken and the safety considerations associated with potential toxicity at high supplemental doses of lutein and zeaxanthin, the use of an appropriate animal model was necessary. Rhesus Macaque primates were chosen for this study because of their similarities to humans; specifically, because of their similar metabolism, and their accumulation of lutein, zeaxanthin, and their metabolites in the serum and macula (Khachik et al., 1995). The challenging task of achieving optimal MPOD in females was posed in this study since MPOD has been shown to be lower in women than in men (Hammond et al., 1996). Thus, female Rhesus Macaques were chosen rather than males. As discussed throughout this thesis, the association between lutein and zeaxanthin and the prevention and treatment of AMD has been implied. Yet, while multi-center clinical trials with these carotenoids are in the planning stage by the NEI, metabolic and toxicity studies with lutein and zeaxanthin had not been conducted prior to this study. For this reason, the experiments outlined in this thesis were undertaken.

RESEARCH QUESTIONS

1) How does long-term chronic supplementation of primates with lutein (9.34 mg/kg body weight and 0.66 mg/kg body weight), zeaxanthin (10 mg/kg body weight), and a 1:1 combination of the two (lutein and zeaxanthin each at a dose of 0.5 mg/kg body weight) affect plasma concentrations and tissue deposition of these carotenoids and their metabolites over time?

2) How is the concentration of lutein, zeaxanthin, and their metabolites in the ocular tissues of primates affected by long-term chronic supplementation with lutein and/or zeaxanthin?

3) Can long-term supplementation of primates with lutein, zeaxanthin, and their combination provide insight into the metabolic pathways of these carotenoids?

4) Can long-term chronic supplementation of primates with pharmaceutical doses of lutein and zeaxanthin result in ocular toxicity and/or side effects? Pharmaceutical doses in this study refer to greater than 60-fold that of the highest amount administered (0.16 mg/kg body weight) in a recent human supplementation study (Moura et al., 2004; Chew et al., 2003)?

METHODS AND ANALYTICAL PROCEDURES

This study was conducted in collaboration with a number of investigators at various institutes of the University of Maryland (Baltimore) and the Wilmer Eye Institute (John Hopkins University, Baltimore) under the supervision of Dr. Frederick Khachik, the principal investigator. The project collaborators and their respective contributions are described herein.

I. Study Investigators

Dr. Steve Shipley (DVM) who is a veterinary pathologist at the Veterinary Resources Division of the School of Medicine oversaw the care and carotenoid supplementation of the primates. The primates were housed at the Medical Student Teaching Facility (MSTF) building of the University of Maryland, Baltimore campus. Additionally, Dr. Shipley was responsible for conducting standard necropsy of all major organs from the sacrificed animals.

All ocular exams were performed by either Dr. Scott Steidl or Dr. Mary Johnson at the University of Maryland, Baltimore School of Ophthalmology who are both board certified ophthalmologists.

Dr. Gerard Lutty and Scott McLeod at the Wilmer Eye Institute at John's Hopkins Medical Center performed complete histopathology of the primate retinas following euthanasia.

The toxicology evaluation was handled by Dr. Jodi Anne Flaws of the Program in Toxicology at the University of Maryland, School of Medicine, Baltimore.

The qualitative and quantitative analysis of lutein, zeaxanthin, and their metabolites as well as vitamin A in the plasma and major organs and tissues (including ocular tissues) of primates were measured in the laboratory of Dr. Khachik (University of Maryland, College Park).

II. Selection of Animal Model for Supplementation Studies with Lutein and Zeaxanthin

Due to the invasive nature of the study and the safety considerations associated with administering pharmaceutical doses of lutein and zeaxanthin, these studies needed to be conducted in an appropriate animal model. Primates were chosen because of their similar metabolism, and their accumulation of lutein, zeaxanthin, and their metabolites in the retina (Khachik et al., 1995). Therefore, female Rhesus Macaques (*Macaca mulatta*) were selected for the chronic supplementation studies with lutein, zeaxanthin, and their combination. This selection was based on the assumption that female Rhesus Macaques, similar to humans, may accumulate a lower macular pigment optical density (MPOD) than their male counterparts. In such a case, establishing a safe dose range at which lutein and zeaxanthin supplementation can result in an optimal MPOD was the challenging task undertaken in these studies.

Study Primates

Eighteen female Rhesus Macaque monkeys weighing between 2.6–3.2 kg were selected and divided into 3 treatment groups designated as L-treated (L at 9.34 mg/kg/day and Z at 0.66 mg/kg/day), Z-treated (Z at 10 mg/kg/day), L/Z-treated (L & Z each at 0.5 mg/kg/day), and a control group. Each treatment group consisted of 5 animals that was

supplemented daily according to their assigned treatment. The control group consisted of 3 primates that were not supplemented and served as the control group for all 3 studies.

Procedures to Assure Comfort and Appropriate Analgesia

The laboratory animal veterinarian (Dr. Steve Shipley) was responsible for the selection of the most appropriate analgesic or anesthetic which best met the clinical and humane requirements without compromising the scientific data. For analgesia, buprenorphine was administered intramuscularly at a dose of 0.015 mg/kg body weight of primate. The Rhesus Macaque monkeys were anesthetized with ketamine at an intramuscular dose of 10 mg/kg body weight. The health of each animal was monitored by performing complete blood chemistry at various intervals throughout the study (Dr. Shipley).

Institutional Animal Care and Use Committee (IACUC) Approval

The proposed studies were approved by the IACUC of the University of Maryland, Baltimore and the University of Maryland, College Park. (See attached letter, Appendix A). Tissue distribution complied with the tenets of the Declaration of Helsinki.

III. Selection of Lutein and Zeaxanthin Doses for the Supplementation Studies

The oral daily doses chosen for the proposed studies, a lutein supplement of 9.34 mg/kg body weight, and a zeaxanthin supplement of 10 mg/kg body weight was approximately 60-fold that of the highest dose of lutein administered in the study conducted by Moura et al. (Moura et al., 2004). These approximately 10 mg/kg of body weight doses of lutein or

zeaxanthin corresponded with approximately 149–303 times the average daily carotenoid intake in the U.S diet (estimated at 2–4 mg/day) (Chug-Ahuja et al., 1993).

The L/Z-treated primates were supplemented daily with a combination of lutein and zeaxanthinin at the dose of 0.5 mg/kg body weight for each of these carotenoids. The lutein dose of 0.5 mg/kg body weight of primates was approximately 3-fold that of the highest dose of lutein supplemented to humans by Moura et al., and the zeaxanthin dose was approximately 10-fold that of the highest zeaxanthin received by subjects in the same study (Moura et al., 2004).

It is relevant to note that the supplementation levels for the Lutein Supplementation Study and Zeaxanthin Supplementation Study are 60-fold that of the highest dose of a recent human supplementation, and thus allowed the examination of potential toxicity as well as the bioavailability at a relatively high level of supplementation (Moura et al., 2004; Chew et al., 2003).

Source of Lutein and Zeaxanthin Supplements

The supplemental doses of lutein and zeaxanthin were formulated by DSM Nutritional Products (Basel, Switzerland) into 5% water-dispersible beadlets. The water-dispersible beadlet formulation is the most bioavailable form of these carotenoids. All supplements were stored in well-sealed aluminum bags within plastic bags at 5°C to protect them from moisture, air, and light. The stability and the composition of the beadlets were monitored by extraction and HPLC analysis throughout the study.

Supplements were given to the animals by adding each dose to a banana and providing it to the animal as a treat.

IV. Study Design

The 3 supplementation studies were designated as the Lutein Supplementation Study, the Zeaxanthin Supplementation Study, and the Lutein/Zeaxanthin Supplementation Study. The Lutein Supplementation Study involved 5 female Rhesus Macaque monkeys (L-treated primates) that were supplemented daily with a high dose of lutein for 12 months. The Zeaxanthin Supplementation Study involved the daily supplementation of 5 female Rhesus Macaque monkeys with a high dose of zeaxanthin for 12 months, and the Lutein/Zeaxanthin Supplementation Study consisted of 5 female Rhesus Macaque monkeys that were supplemented daily with a 1:1 combination of lutein and zeaxanthin, each at the dose of 0.5 mg/kg for 12 months. The control group (n = 3) was not fed any supplements.

Prior to this study, neither long-term high-dose supplementation with lutein or zeaxanthin nor the effects of supplementation with a combination of lutein and zeaxanthin in primates or humans had been investigated. The proposed study was designed to investigate the safety and efficacy of high-dose supplementation with lutein or zeaxanthin, and the effects of combined lutein and zeaxanthin supplementation on plasma and tissue carotenoid levels as well as the combined protective effect of these carotenoids against AMD.

At baseline, months 6, 12, and 18 plasma samples were collected for the analysis of carotenoids and their metabolites. Major organs and tissues (liver, lung, colon, breast, kidney, spleen, ovary, cervix, adipose) including ocular tissues (retina, ciliary body, lens, iris) of the sacrificed animals were analyzed for carotenoids and their metabolites. Additionally, the primates' weights were recorded at baseline and at months 6, 12, and 18.

Several other biomarkers were analyzed by other co-investigators of this study including urinary creatinine and proteins, histopathology of retinas, and ocular exams that included fundus examination and photography, as well as multifocal ERGs. The abovementioned urinary analyses and ocular assessments were performed at baseline, months 6, 12, and 18. Complete histopathology of the retina and *in vitro* culture of primate keratinocyte and retinal cells were completed at either month 12 or month 18 depending on which time point the primate was sacrificed.

The Control Group

The control group consisted of 3 female Rhesus monkeys that were housed with the Lutein Supplementation Study animals and served as the control group for the entire study. These animals were fed a standardized monkey diet, which is described later in this section, and was identical to that fed to the other study primates. The collection of biomarkers for the control group followed the same timeline as that of the animals in the Lutein Supplementation Study. One control primate was sacrificed at month 12 and the remaining 2 were sacrificed at month 18.

The Lutein Supplementation Study

A total of 5 female Rhesus monkeys comprised the treatment group. The L-treated primates of the Lutein Supplementation Study received daily lutein supplements at the dose of 10 mg/kg for the duration of 12 months. The lutein in these supplements contained approximately 6.6% zeaxanthin by weight so each 10 mg dose consisted of

approximately 9.34 mg of lutein and 0.66 mg of zeaxanthin. Therefore, the L-treated primates actually received 9.34 mg/kg of lutein and 0.66 mg/kg of zeaxanthin daily.

Due to the high supplemental dose given to the primates in the L-treated group, statistically significant differences in measurements of the various biomarkers at the months 6 and 12 of supplementation were expected. At the end of the supplementation period, 2 of the L-treated primates were sacrificed and the remaining 3 animals remained under observation for 6 months to assess whether there was any toxicity associated with these high supplemental doses. At month 18, the remaining L-treated primates were sacrificed.

The Zeaxanthin Supplementation Study

A total of 5 female Rhesus Macaque monkeys were supplemented daily with zeaxanthin at the dose of 10 mg/kg body weight for 12 months. At the end of the supplementation period (month 12), 2 of the Z-treated primates were sacrificed and the remaining 3 animals were kept under observation for 6 months. After 6 months of post-supplementation observation (month 18), the remaining 3 Z-treated primates were sacrificed.

The Lutein/Zeaxanthin Supplementation Study

A total of 5 female Rhesus Macaques monkeys were supplemented daily with a 1:1 mixture of lutein and zeaxanthin to provide a lutein dose of 0.5 mg/kg body weight and a zeaxanthin dose of 0.5 mg/kg body weight for 12 months. All 5 of the L/Z-treated primates were sacrificed after 12 months of supplementation.

V. Collection, Handling, and Shipping of the Primate Plasma and Tissue Samples for Measurement of Biomarkers

Collection of Plasma Samples for Carotenoid Analysis

Two fasting blood samples, 10 mL each, were drawn from each primate via venipuncture at the specified intervals for these studies. The collected blood samples were immediately placed on ice, protected from light, and centrifuged (1000 x *g*) for 20 minutes at 4°C within 1 hour after collection to separate the plasma. Approximately 4–5 ml of plasma was expected from each 10 ml blood sample drawn from each primate.

The plasma samples were dated and coded with the appropriate number for each primate known only to the study coordinator in Baltimore (Dr. Shipley) and stored at -70°C or lower. The samples for carotenoid analysis were transported to our laboratories on Dry Ice for analysis. Upon arrival, the plasma samples were immediately stored at -70°C; the samples were extracted and analyzed by HPLC as promptly as possible.

Method of Euthanasia

The Rhesus Macaque monkeys were euthanized at the end of the study. The animals were first anesthetized with an intramuscular dose of ketamine of 10 mg/kg body weight. The animals were then humanely euthanized with a 100 mg/kg dose of pentobarbitol injected intravenously consistent with the 1993 Report of the American Veterinary Medical Association panel on euthanasia. The procedures were performed and monitored by Dr. Shipley and his colleagues.

Collection, Handling, and Shipping of Tissues and Organs

At sacrifice, all tissues and organs to be analyzed were removed, dated, and coded with the appropriate number for each primate known only to the study coordinator at Baltimore (Dr. Shipley) and stored at -70°C or lower until they were transported on Dry Ice to our laboratories (UMD, College Park) for analysis.

One of the eyes from each primate was dissected under the supervision of Dr. Steidl or Dr. Johnson and the various ocular tissues were stored at -70°C or lower. The dissected eye tissues of one eye from each animal was labeled and coded with the appropriate number for each primate known only to the study coordinator at Baltimore (Dr. Shipley) and stored at -70°C or lower until they were shipped via Express Mail on Dry Ice to our laboratories (UMD, College Park) for analysis.

The other intact eye from each primate was labeled and coded with an appropriate number for each primate and stored at -70°C or lower until they were transported to Dr. Gerard Lutty (Wilmer Eye Institute, JHU, Baltimore) for histhopathology.

Collection of Urine for Analysis of Urinary Creatinine and Proteins

Urine samples for creatinine and protein analyses were collected by placing the animals in metabolic cages with free access to water for 24-hour urine collections. The urine samples was collected in foil-wrapped containers containing sodium carbonate, 24-hour urine volumes was measured and recorded prior to being frozen at –80°C.

VI. Measurement of Biomarkers

Biomarkers of Toxicity

Specific biomarkers associated with skin, liver and kidney function were probed as these organs are actively involved in the metabolism and excretion of various organic substances and tend to concentrate dietary carotenoids. The 3 toxicological evaluations that were conducted by Dr. Flaws included: 1) culturing of skin epithelial cells and consequent examination for any alterations in gene expression patterns; 2) urinary creatinine excretion patterns and potential hepatoxic effects; and 3) identification of chemical-specific urinary proteins using N-terminal sequence analysis which was performed by Dr. Flaws of the University of Maryland, School of Medicine, Baltimore.

Ocular Toxicity

Possible retinopathy was monitored throughout the supplementation trials. Whenever the monkeys' eyes were dilated, a board certified ophthalmologist (Dr. Steidl or Dr. Johnson) examined the retinas with indirect ophthalmoscopy. Any detected abnormalities were recorded using Fundus photography. Histopathology of the retina was performed by Dr. Gerard Lutty and Scott McLeod (Wilmer Eye Institute). Dr. Mary Johnson also performed multifocal, scotopic, and photopic electroretinography. At various intervals (baseline, months 6, 12 and 18) multifocal ERGs was taken from the monkeys to monitor for any electrophysiological evidence of carotenoid-induced toxicity.

Primate Plasma Analysis

Plasma that was collected from each of the treatment and control primates at the various intervals (baseline, months 6, 12, and 18) was sent to our laboratories for

qualitative and quantitative analysis (UMD, College Park). Normal phase HPLC-UV/visible-photodiode array detection was employed for identification and quantification of lutein, zeaxanthin and related geometrical isomers, as well as metabolites and retinol. The procedures for plasma carotenoid extraction and HPLC analysis is described later in this section.

Primate Tissue Analysis

The qualitative and quantitative distributions of carotenoids in major organs and tissues [adipose, cervix, colon, kidney, liver, lung, breast, spleen, ovaries] as well as ocular tissues (retina, iris, lens, ciliary body) of the necropsied primates were determined in our laboratories (UMD, College Park) by extraction and HPLC analysis.

Normal phase HPLC-UV/visible photodiode array detection was employed for identification and quantification of lutein, zeaxanthin and related geometrical isomers as well as their metabolites and retinol. HPLC-UV/visible photodiode array detection on a chiral column was employed for the identification and quantification of (3R,3'R)-zeaxanthin and (3R,3'S; *meso*)-zeaxanthin in the retina tissues. These procedures are described later in this thesis.

VII. Standardized Monkey Diet

The standardized monkey diet was purchased in bulk once every 6 weeks from Harlan Tekland (Madison, WI). The feed chosen as the standard diet, Harlan Tekland-8775, contained the highest concentrations of lutein and zeaxanthin available (total lutein content: 5.49 μg/g feed; total zeaxanthin content: 1.47 μg/g feed). Prior to the beginning

of the supplementation period of these studies, a sample of this feed (Harlan Telkand-8775) was extracted and analyzed by HPLC to determine the carotenoid levels of the diet.

All primates in the study were kept on the same standard monkey diet. The diet came in the form of biscuits, each weighing approximately 12 grams. Each monkey ate between 15 and 20 monkey biscuits per day equaling 180 to 240 grams of feed. This amount of feed supplied the primates with between 0.99 and 1.32 mg of lutein, and 0.26 and 0.35 mg of zeaxanthin per day. The light cycle for all the animals was maintained at 12 hours of light and 12 hours of dark.

VIII. Extraction and Analysis of Lutein and Zeaxanthin Beadlets

A suspension of 300 mg of the beadlets of lutein or zeaxanthin and approximately 100 mg of protease Maxatase P 440,000 (DSM Nutritional Products, Basel, Switzerland) in 10 mL of deionized water in a 100 mL volumetric flask was sonicated in an ultrasonic water bath for 30 minutes. Ethanol (30 mL) and dichloromethane (40 mL) were added and the mixture was sonicated for 10 minutes. The solution was brought up to a final volume of 100 mL using dichloromethane, and after mixing, the solids were allowed to settle. A 1 mL aliquot of the solution was transferred into a vial and evaporated to dryness under nitrogen. The residue was dissolved in the HPLC eluent (a 3:1 mixture of hexane:dichloromethane) and filtered through a 0.45 μm disposable polyvinylidene fluoride filter assembly (Acrodisk; VWR Scientific products, Bridgeport, NJ) into a 25 mL volumetric flask and brought up to volume for HPLC analysis on a silica-based nitrile bonded column according to our published procedures (Khachik et al., 1992; Khachik et al., 1997a;). For spectrophotometric analysis, a 1 mL aliquot of the solution of lutein or

zeaxanthin was similarly filtered into 25 mL volumetric flask and brought up to volume using ethanol. The concentrations of lutein (λ_{max} = 445 nm, $E^{1\%}$ = 2550) and zeaxanthin (λ_{max} = 450 nm, $E^{1\%}$ = 2540) in the extracts were measured in ethanol at their corresponding absorption maximum and extinction coefficient (Britton, 1995).

IX. Extraction of Carotenoids from Primate Plasma

All primate plasma samples were shipped from the University of Maryland, Baltimore to the laboratory of Dr. Khachik on dry ice. Upon receipt, primate plasma samples were catalogued and stored in a freezer at -80°C. Primate plasma samples were first thawed, and accurately measured using a disposable syringe (latex-free syringe, 5 mL, VWR Scientific Products) and then transferred into a 50 mL centrifuge tube (Blue Max™, polypropylene conical tube). Each sample was treated with 5 mL ethanol to precipitate the proteins. The plasma carotenoids were extracted by adding tetrahydrofuran (THF) containing 0.1 % 2,6-di-*tert*-butyl-4-methylphenol (BHT); the amount of THF added was equal to the plasma volume plus the volume of ethanol previously added. The tube was then vortexed for 2 minutes, and then centrifuged at 2000 *g* for 5 minutes to separate the protein solids from the supernatant liquid. The extract was removed with a pipette and saved, and the solid was reextracted by adding 5 mL of THF (with 0.1% BHT) to the protein residue and then repeating the vortexing and centrifuging processes as outlined above. The combined extracts were evaporated to dryness using an evaporator (RapidVap Vacuum, model 79000-02, LABCONCO Co., MO). The dried extract was dissolved in dichloromethane, sonicated, centrifuged, and then filtered through a 0.45 µm disposable polyvinyl fluoride filter (VWR, Scientific Products, NJ) into a 5mL graduated

micro-sample vial. The solvent was evaporated under nitrogen (Nitrogen Evaporator, N-EVAP™ 112, Organomation Associates, Inc., MA) and 250 µL of the HPLC injection solvent (hexane 75%, dichloromethane 25%, methanol 0.35%, *N*,*N*-diisopropylethylamine 0.10%) was added to the dried extract. The vial was then sonicated and centrifuged. For normal phase HPLC, 120 µL of the extract was transferred to a chromatography vial and 50 µL was injected into the HPLC system. The remaining extract was stored at -80°C in the event that the HPLC analysis needed to be repeated.

X. Extraction of Carotenoids from Primate Tissues

All primate tissue samples were shipped on dry ice to Dr. Khachik's laboratory (UMD, College Park). Upon receipt, primate tissue samples were stored in a freezer at -80°C. Tissue samples were thawed to room temperature and blood was removed by washing with water; the excess water was removed by blotting the tissue samples with paper towels and applying gentle pressure with a mortar. Each tissue sample was cut into smaller pieces to facilitate the extraction of carotenoids by increasing the surface area. Samples were accurately weighed and then placed into a beaker and anhydrous sodium sulfate (10% by weight of the tissue) and sufficient volume of tetrahydrofuran (THF) containing 0.1 % BHT to completely cover the sample was then added. The samples were sonicated for 1 hour in an ice bath (10–15°C) and the extract was then decanted into a round bottom flask. The tissue was reextracted by adding more THF, crushing the sample with a mortar, and then sonicating for 30 minutes. The extracts were combined and filtered on a Buchner funnel. The solids were washed with THF and the filtrate was transferred to the round bottom flask and combined with previously saved extracts. The

combined extract was then evaporated on a rotary evaporator at 40°C. The round bottom flask was thoroughly rinsed with dichloromethane, sonicated to remove the residual sample from the sides of the flask, and the extract was then filtered through a 0.45 µm disposable polyvinyl fluoride filter (VWR, Scientific Products, NJ) and diluted to an appropriate volume. The solvent was evaporated to dryness under nitrogen (Evaporator, N-EVAP™ 112, Organomation Associaties, Inc., MA). An appropriate volume of HPLC injection solvent was added to the residue and depending upon the carotenoid concentration, the sample was diluted to a final volume of 0.3, 1.0, 3.0, or 5.0 mL. The extract (120 µL) was transferred to a chromatography vial and 50 µL was injected into the HPLC system. The remaining sample was stored at -80°C in the event that additional HPLC analysis was required.

XI. HPLC Analysis of Carotenoids

Carotenoid Separation by HPLC Normal Phase Column

Plasma and tissue extracts were analyzed using a normal phase HPLC system according to a published procedure (Khachik et al., 1997b). HPLC separations were carried out on a silica-based nitrile bonded HPLC column (Spherisorb®; 250 mm length x 4.6 mm i.d.; 5 µm spherical particle; Waters Company, U.S.). The column was protected with a nitrile-bonded guard cartridge (3 cm length x 4.6 mm i.d.; 5 µm spherical particle). The mobile phase consisted of an isocratic mixture of hexanes (75%), dichloromethane (25%), methanol (0.35%), and *N*,*N*-diisopropylethylamine (DIPEA, 0.1%). The column flow rate was 0.8 ml/min. HPLC runs were simultaneously monitored at 325, 446, and 456 nm. Carotenoids were identified by comparison of their HPLC retention times and

UV-visible absorption spectra with those of authentic standards. Carotenoids were quantified from calibration tables obtained from the HPLC response factors of standards at five or six known concentrations using linear regression analysis.

For the retina tissues, zeaxanthin concentrations were initially determined using a normal phase column, but because dietary zeaxanthin and nondietary *meso*-zeaxanthin cannot be separated by this method, further HPLC analysis of the extracts on a chiral column was necessary.

Carotenoid Separation of Retina Tissue Extracts by HPLC Chiral Column

Retina extracts were also analyzed on a chiral HPLC column according to a previously published procedure (Khachik et al., 2003). HPLC separations were carried out on a [amylose tris-(3,5-dimethylphenyl-carbamate) coated on 10 µm silica gel] chiral HPLC column. This allowed for the simultaneous separation of (3R,3'R)-zeaxanthin, (3R,3'S; *meso*)-zeaxanthin, (3S,3'S)-zeaxanthin, (3R,3'R,6'R)-lutein, and 3'-epilutein. This analysis was not performed on the ciliary body, iris, or lens tissues because the concentrations of zeaxanthin and *meso*-zeaxanthin were too low for chiral HPLC analysis. Therefore, only the total concentration of (3R,3'S; *meso*)-zeaxanthin and dietary (3R,3'R)-zeaxanthin were reported.

XII. Accuracy and Reproducibility of HPLC Results

The accuracy of extractions was monitored regularly by the extraction, identification, and quantification of carotenoids in standardized Red Cross plasma. No internal standard in the extraction and analysis of various samples was employed because

of the possibility that its HPLC peak could interfere with the presence of possible unknown carotenoids.

The reproducibility of the normal phase HPLC analysis of carotenoids was monitored regularly by HPLC analysis of a calibrated solution containing known concentrations of (3R,3'R,6'R)-lutein, (3R,3'R)-zeaxanthin, and 3'-epilutein.

XIII. Statistical Analysis

Statistical analysis of the data obtained from plasma and ocular tissue carotenoid analyses were performed by Analysis of Variance and Covariance (ANCOVA) with repeated measurements using SAS version 8.2 (SAS Institute Inc., Cary, NC). The least significant difference (LSD) protected test was used to determine which treatment groups' mean plasma and ocular tissue levels of lutein, zeaxanthin, and their metabolites were significantly different from one another at baseline and months 6, 12, and 18. Similarly, the interactions between the body weight or age of the animals and the plasma lutein and zeaxanthin levels were also examined. A p value of less than 0.05 was considered statistically significant.

RESULTS

Lutein Supplementation Study: Daily Supplementation of Female Rhesus Macaque Monkeys with Lutein (9.34 mg/kg L, 0.66 mg/kg Z) for 12 Months

Plasma Carotenoid Analysis of Primates in the Lutein Supplementation Study

The plasma of 3 control and 5 L-treated primates was collected at baseline, month 6, and month 12 of the 12-month supplementation period. The control and treatment groups ate the same standardized monkey diet which provided each primate with 0.99–1.32 mg of lutein and 0.26–0.35 mg of zeaxanthin per day. The bioavailability of these carotenoids in the feed was not known. At month 12, one control and 2 L-treated primates were euthanized. The remaining animals were kept under observation for 6 months after the end of the supplementation period and then sacrificed. Plasma samples from these primates were also taken at month 18, at the conclusion of the 6-month post-supplementation period. All plasma samples were analyzed for lutein, zeaxanthin, their metabolites, and retinol.

The mean plasma lutein levels for primates in the control group at baseline (0.244 ± 0.021 µmol/L) compared to the baseline values for the primates in the L-treated group (0.223 ± 0.023 µmol/L) were not significantly different ($p = 0.84$). The data for plasma lutein concentrations are shown in Table 1. The mean plasma lutein concentration for the control group at baseline was not significantly different compared to the mean levels measured at month 6 (0.235 ± 0.024 µmol/L; $p = 0.89$) and month 12 (0.228 ± 0.041 µmol/L; $p = 0.84$). However, the mean plasma lutein level of the L-treated group at baseline (0.223 ± 0.023 µmol/L) was significantly different compared to the mean levels at month 6 (0.573 ± 0.079 µmol/L; $p < 0.001$) and at month 12 (0.709 ± 0.101 µmol/L; $p < 0.001$) (Figure 4). Mean plasma lutein levels for the L-treated group were also significantly different ($p = 0.01$) between 6 and 12 months. The mean plasma lutein level for the L-treated

primates at month 18 (0.227 ± 0.05 µmol/L) was not significantly different than their mean baseline levels or than the mean levels of the control primates.

Table 1. Plasma lutein concentrations (µmol/L, mean ± SEM)* of the control and the L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) at baseline, months 6, 12, and 18.

Primate ID	Total Lutein Concentration in Plasma (µmol/L)†			
	Baseline	**6 Months**	**12 Months**	**18 Months**
Control Group				
RQ4107	0.253	0.280	0.309	0.257
RQ4142	0.275	0.227	0.181	0.166
RQ4079	0.205	0.199	0.194	--‡
Mean ± SEM	**0.244 ± 0.021[a]**	**0.235 ± 0.024[a]**	**0.228 ± 0.041[a]**	**0.212 ± 0.046[a]**
Treatment Group				
RQ4087	0.122	0.315	0.411	--‡
RQ4078	0.253	0.801	0.961	0.250
RQ4122	0.253	0.582	0.798	--‡
RQ4189	0.241	0.524	0.537	0.135
RQ4175	0.247	0.644	0.838	0.295
Mean ± SEM	**0.223 ± 0.084[a]**	**0.573 ± 0.062[b]**	**0.709 ± 0.070[c]**	**0.227 ± 0.052[a]**

* SEM = standard error of the mean; † Total lutein refers to the combined concentrations of *all-trans*-lutein and its *cis*-isomers in plasma; ‡ Primate sacrificed at the end of 12-month supplementation period; Values with different letters (a, b, c) denote statistical significance across each row and within each column

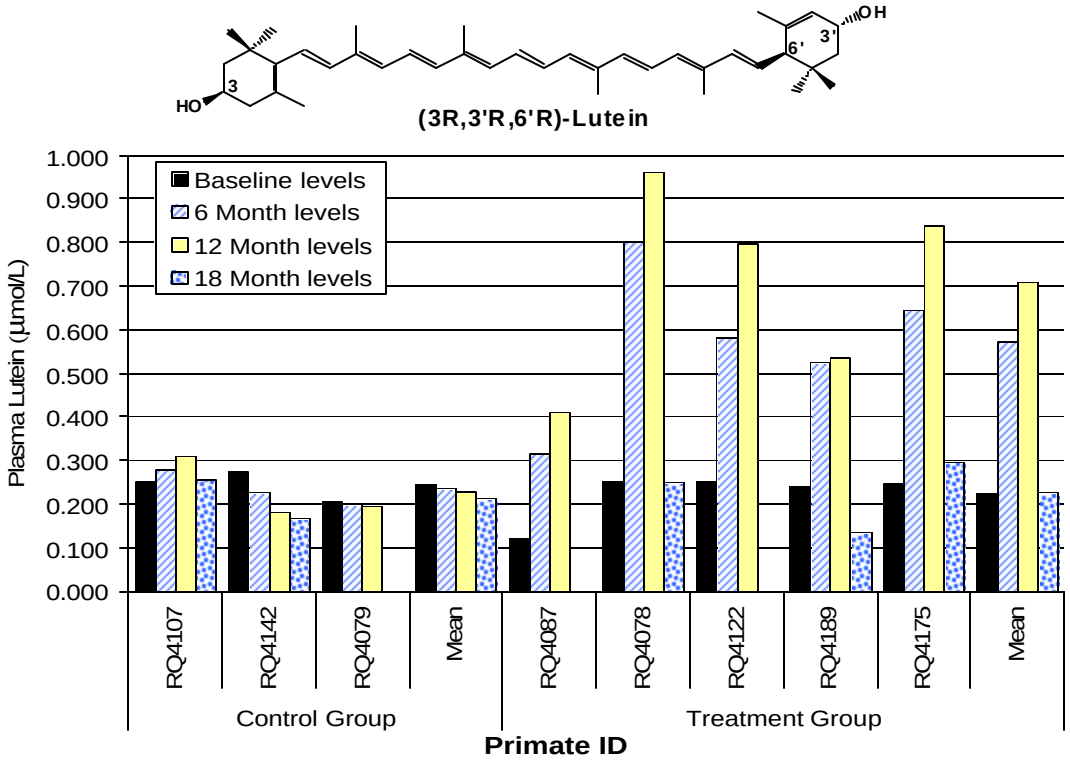


Figure 4. Changes in the plasma concentrations of lutein (µmol/L) in the control and the L-treated primates during the 12-month supplementation period and 6 months post-supplementation.

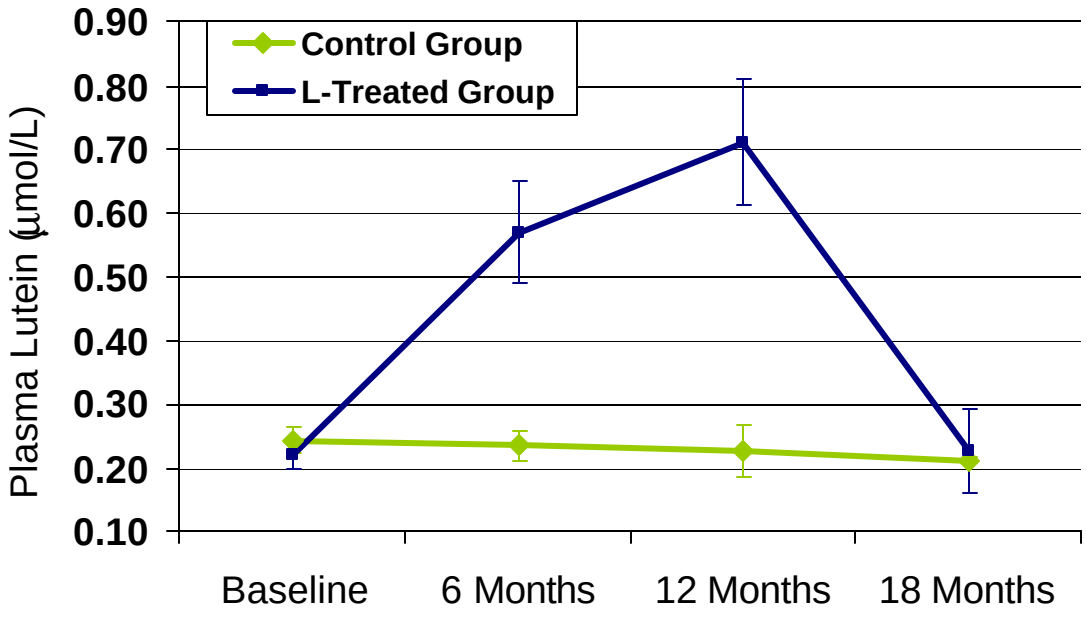


Figure 5. Changes in the mean (±SEM) plasma concentrations of lutein (µmol/L) for the L-treated group (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) and the control group at baseline, months 6, 12, and 18.

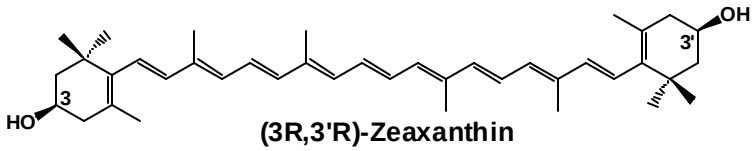
It is critical to note that the lutein supplements given to the primates contained approximately 6.6% zeaxanthin, thus supplying the L-treated primates with a daily dose of 9.34 mg/kg (16.42 µmol/kg) of lutein plus 0.66 mg/kg (1.16 µmol/kg) of zeaxanthin. Extraction and HPLC analysis of lutein (and zeaxanthin) beadlets revealed no significant changes in quantitative and qualitative profiles throughout the study. As shown in Table 2 and Figure 6, the plasma concentrations of zeaxanthin in the L-treated group increased during the 12-month supplementation period. The mean plasma zeaxanthin level at baseline for primates in the control group (0.118 ± 0.009 µmol/L) compared with the mean baseline value for the primates in the L-treated group (0.111 ± 0.018 µmol/L) was not significantly different ($p = 0.80$) (Figure 7). At baseline, the mean plasma zeaxanthin level for the control group (0.118 ± 0.009 µmol/L) was not significantly different from

the mean levels measured at 6 months (0.069 ± 0.010 µmol/L; p = 0.05) and 12 months (0.120 ± 0.027 µmol/L; p = 0.92). For the L-treated group, the mean plasma zeaxanthin level at baseline (0.111 ± 0.018 µmol/L) was not significantly different than the mean level at six months (0.123 ± 0.018 µmol/L; p = 0.09), but was significantly lower (p < 0.001) than the mean plasma level at 12 months (0.147 ± 0.018 µmol/L). The mean plasma zeaxanthin levels were also significantly different (p = 0.005) between months 6 and 12. At the end of month 18, 6 months post-supplementation, the mean plasma zeaxanthin level of the L-treated group (0.107 ± 0.027 µmol/L) had nearly returned to its baseline level (0.111 ± 0.018 µmol/L).

Table 2. Plasma zeaxanthin concentrations (µmol/L) in the control and the L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) at baseline, months 6, 12, and 18.

Primate ID	**Total Zeaxanthin Concentrations in Plasma (µmol/L)***			
	Baseline	**6 Months**	**12 Months**	**18 Months**
Control Group				
RQ4107	0.133	0.081	0.174	0.147
RQ4142	0.129	0.079	0.094	0.095
RQ4079	0.092	0.049	0.091	--†
Mean ± SEM[b]	**0.118 ± 0.009**[a]	**0.069 ± 0.010**[a]	**0.120 ± 0.027**[a]	**0.120 ± 0.026**[a]
Treatment Group				
RQ4087	0.061	0.058	0.840	--†
RQ4078	0.116	0.142	0.167	0.132
RQ4122	0.136	0.161	0.186	--†
RQ4189	0.129	0.119	0.141	0.070
RQ4175	0.111	0.133	0.155	0.118
Mean ± SEM	**0.111 ± 0.018**[a]	**0.123 ± 0.018**[a]	**0.147 ± 0.018**[b]	**0.107 ± 0.027**[a]

* Total zeaxanthin refers to the combined concentrations of *all-trans*-zeaxanthin and its *cis*-isomers in plasma; † Primate sacrificed at the end of 12-month supplementation period; ‡ SEM = standard error of the mean; Values with different letters (a, b) denote statistical significance across each row and within each column



(3R,3'R)-Zeaxanthin

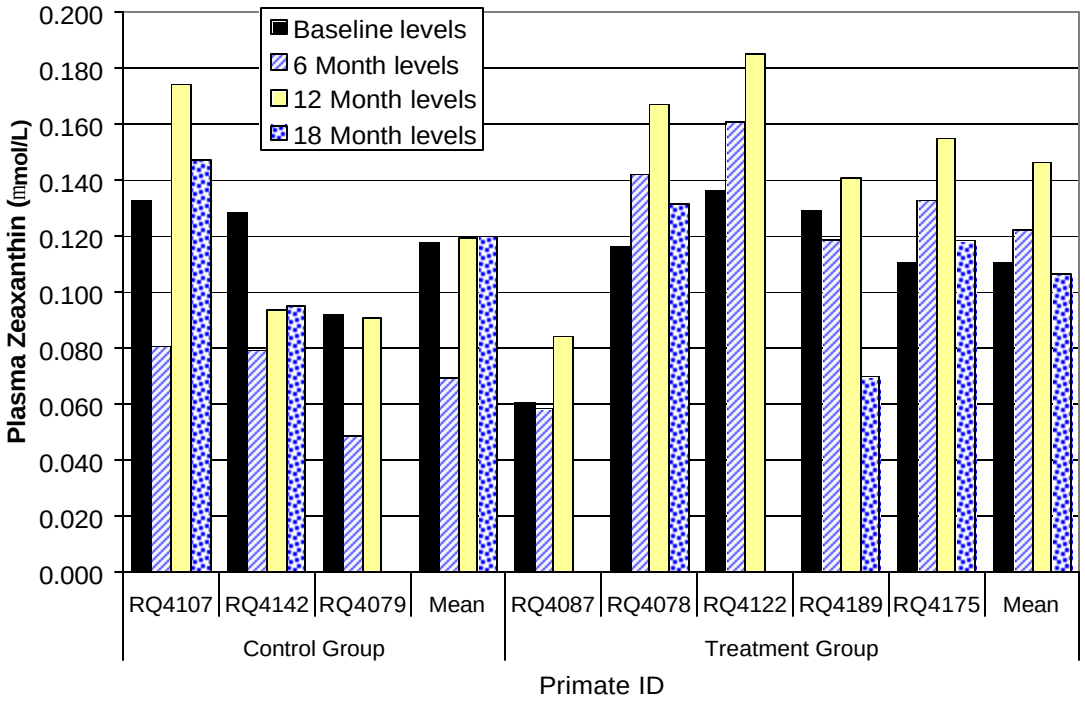


Figure 6. Changes in plasma concentrations of zeaxanthin (µmol/L) in the L-treated group (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) and the control group at baseline, months 6, 12, and 18.

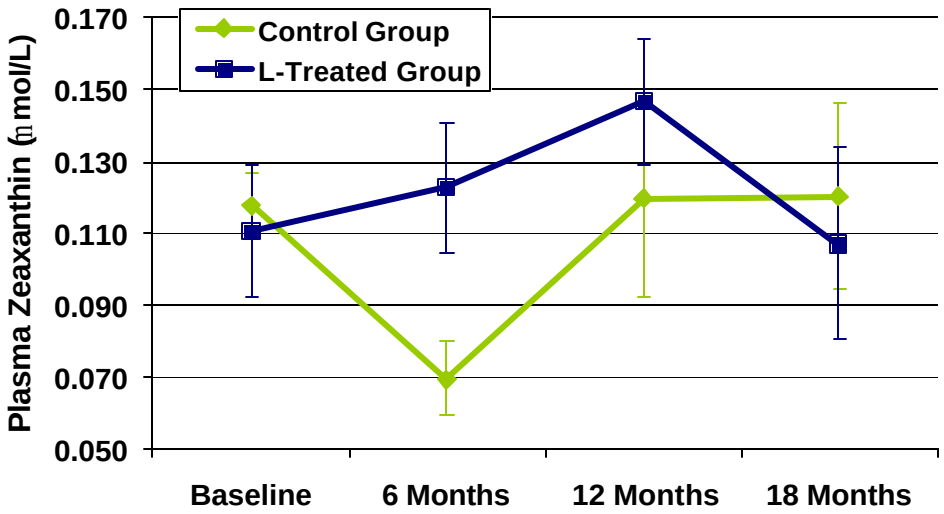


Figure 7. Changes in the mean plasma concentrations of zeaxanthin (µmol/L) in the L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) and the control primates at baseline, months 6, 12, and 18.

Plasma Concentrations of Lutein and Zeaxanthin Metabolites in Primates in the Lutein Supplementation Study

Two of the lutein and zeaxanthin metabolites analyzed in the plasma of the L-treated primates were (3R,3'S,6'R)-lutein (3'-epilutein) and 3-hydroxy-β,ε–caroten-3'-one (3'-oxolutein). The structures of these metabolites are shown in Figure 8, and the proposed pathway for their *in vivo* formation is depicted in Figure 2. Plasma concentrations of these carotenoids were measured at baseline, months 6 and 12, and also at 6 months post-supplementation (month 18) in the primates that were kept under observation after the supplementation period (n = 3).

The data for 3'-epilutein plasma levels, presented in Table 3, show that the mean plasma 3'-epilutein level for the control group at baseline (0.012 ± 0.0012 μmol/L) and the mean baseline level for the L-treated group (0.013 ± 0.0028 μmol/L) were not significantly different (p = 0.92). Additionally, the mean plasma 3'-epilutein level for the control group at baseline compared to that at 6 months (0.0073 ± 0.0021 μmol/L) was not significantly different (p = 0.13), nor was the mean baseline value significantly different (p = 0.29) from the mean level for the control group at month 12 (0.0089 ± 0.0021 μmol/L). See Figure 9 for a comparison of the mean 3'-epilutein levels for the control and the L-treated primates. While the mean plasma 3'-epilutein levels for the L-treated group were not significantly different between baseline (0.0173 ± 0.0028 μmol/L) and month 6 (0.0182 ± 0.0042 μmol/L; p = 0.60), the mean 3'-epilutein level at month 12 (0.0423 ± 0.0113 μmol/L) was significantly higher (p = 0.01) than the mean level at baseline. For the L-treated group, the mean plasma concentrations of 3'-epilutein between months 6 and 12 were also significantly different (p = 0.03). The mean plasma 3'-epilutein level for the L-treated group at month 18 had returned to near the mean baseline level.

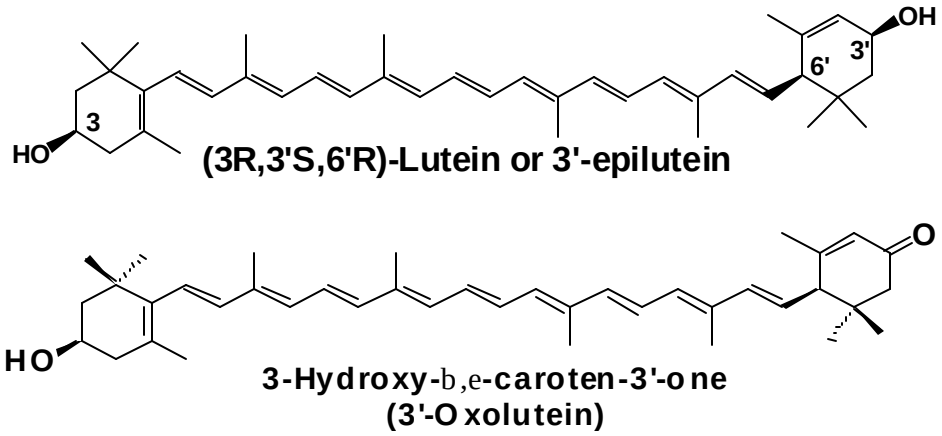


Figure 8. Lutein and zeaxanthin metabolites measured in the plasma of the study primates: (3R, 3'S, 6'R)-lutein (3'-epilutein) and 3-hydroxy-β,ε–caroten-3'-one (3'-oxolutein).

Results from plasma carotenoid analysis showed an increase in 3'-oxolutein levels in the L-treated primates. 3'-Oxolutein is presumably formed from *in vivo* oxidation of dietary or supplemental lutein. The data for 3'-oxolutein levels in L-treated primates compared to the control primates are shown in Table 4.

Table 3. Plasma concentrations (µmol/L) of 3'-epilutein in the L-treated group (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) and the control group at baseline, months 6, 12, and at 6 months post-supplementation (month 18).

Primate ID	3'-Epilutein Concentration in Plasma (µmol/L)			
	Baseline	**6 Months**	**12 Months**	**18 Months**
Control Group				
RQ4107	0.0106	0.0113	0.0093	0.0104
RQ4142	0.0139	0.0070	0.0051	0.0086
RQ4079	0.0114	0.0037	0.0123	--[†]
Mean ± SEM[b]	**0.0120 ± 0.001[a]**	**0.0074 ± 0.002[a]**	**0.0090 ± 0.002[a]**	**0.0095± 0.001[a]**
Treatment Group				
RQ4087	0.0060	0.0051	0.0204	--[†]
RQ4078	0.0090	0.0185	0.0410	0.0090
RQ4122	0.0211	0.0150	0.0835	--[†]
RQ4189	0.0102	0.0312	0.0236	0.0102
RQ4175	0.0173	0.0208	0.0431	0.0173
Mean ± SEM	**0.0127 ± 0.003[a]**	**0.0181 ± 0.004[a]**	**0.0422 ± 0.011[b]**	**0.0122 ± 0.003[c]**

[†] Primate sacrificed after 12 months of supplementation; [‡] SEM = standard error of mean; Values with different letters (a, b, c) denote statistical significance across each row and within each column.

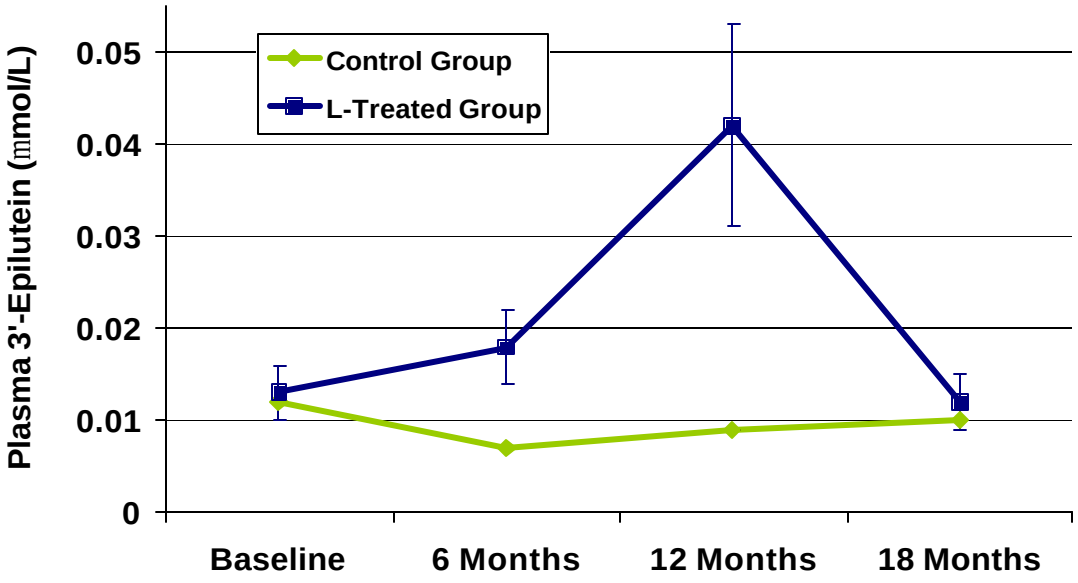


Figure 9. Changes in the mean plasma concentrations of 3'-epilutein in the control and the L-treated primates at baseline, months 6, 12, and 18.

At baseline, the mean plasma concentration of 3'-oxolutein for primates in the control group (0.041 ± 0.01 µmol/L) compared with the mean value at baseline for the

primates in the treatment group (0.029 ± 0.004 µmol/L) was not significantly different (p = 0.49) (Figure 10). Also, the mean concentrations of 3'-oxolutein for the control group at baseline and at month 6 (0.0334 ± 0.010 µmol/L) were not significantly different (p = 0.59) nor were the mean values at baseline and month 12 (0.0327 ± 0.010 µmol/L; p = 0.56). However, the mean concentration of 3'-oxolutein for the primates in the L-treated group at baseline (0.029 ± 0.004 µmol/L) was significantly lower than the mean levels at month 6 (0.071 ± 0.012 µmol/L; p = 0.001) and month 12 (0.085 ± 0.012 µmol/L; p < 0.0010). However, the mean concentrations of 3'-oxolutein in the plasma of the L-treated primates were not significantly different (p = 0.19) between months 6 and 12 of the supplementation period. The mean plasma 3'-oxolutein level for the treatment group 6 months after the end of the supplementation period (0.0319 ± 0.009 µmol/L) returned to the mean baseline level (0.0290 ± 0.005 µmol/L).

Table 4. Plasma concentrations (µmol/L) of 3'-oxolutein in the control group and the L-treated group (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) at baseline, months 6, 12, and at 18.

	3'-Oxolutein Concentration in Plasma (µmol/L)			
Primate ID	**Baseline**	**6 Months**	**12 Months**	**18 Months**
Control Group				
RQ4107	0.0625	0.0291	0.0521	0.0338
RQ4142	0.0362	0.0518	0.0239	0.0198
RQ4079	0.0224	0.0189	0.0223	--[a]
Mean ±SEM[b]	**0.0405 ± 0.012**[a]	**0.0334 ± 0.010**[a]	**0.0327 ± 0.010**[a]	**0.0268 ± 0.00**[a]
Treatment Group				
RQ4087	0.0157	0.0309	0.0505	--[a]
RQ4078	0.0247	0.0758	0.0891	0.0281
RQ4122	0.0353	0.0627	0.1168	--[a]
RQ4189	0.0272	0.0819	0.0237	0.0191
RQ4175	0.0417	0.1005	0.0637	0.0484
Mean ± SEM	**0.0290 ± 0.004**[a]	**0.0714 ± 0.012**[b]	**0.0852 ± 0.012**[b]	**0.0319± 0.009**[a]

[a] Primate sacrificed after 12 month supplementation period; [b] SEM = standard error of mean; Values with different letters (a, b, c) denote statistical significance across each row and within each column.

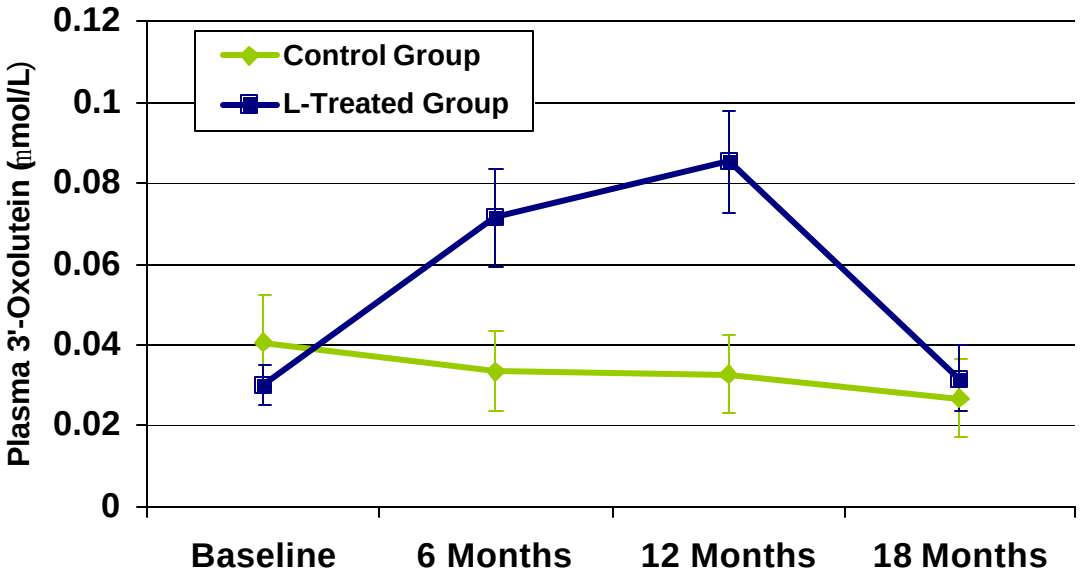


Figure 10. Changes in the mean plasma concentrations of 3'-oxolutein (µmol/L) in the L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) and the control primates at baseline and months 6, 12, and 18.

Carotenoid Concentrations in the Ocular Tissues of Primates in the Lutein Supplementation Study

After 12 months of supplementation, one primate from the control group and 2 primates from the group supplemented daily with lutein (9.34 mg/kg) and a lower dose of zeaxanthin (0.66 mg/kg) were sacrificed. The ocular tissues (retina, ciliary body, iris, lens) from one eye of each animal were extracted and analyzed for carotenoids and their metabolites (Table 5). Six months after the end of the supplementation period (month 18), the remaining 2 control and 3 L-treated primates were sacrificed. The ocular tissues described above from one eye of each animal were similarly extracted and analyzed for carotenoids and their metabolites. In addition to 3'-oxolutein, *meso*-zeaxanthin, a metabolite of lutein that is absent in plasma was also measured in the retinas of the primates. Because the primates in the control group were not supplemented with lutein

and/or zeaxanthin, the mean values for carotenoid concentrations in the ocular tissues of all 3 animals were used for the statistical analysis. The data for the mean values of each group are shown in Table 5 and the complete data for each primate are included as Appendix B.

Table 5. Concentrations (ng/tissue; mean ± SEM) of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein in the ocular tissues of 2 L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) at month 12, and 3 L-treated primates at month 18 in comparison with the mean levels for the control primates (n = 3).

Primate ID	**Concentrations (ng/tissue) of Carotenoids and Their Metabolites in Primates Ocular Tissues** *								
	Retina				**Ciliary Body**			**Iris**	**Lens**
	lutein	**zea-xanthin**	***meso*-zea-xanthin**	**3'-oxo-lutein**	**lutein**	**zea-xanthin**	**3'-oxo-lutein**	**lutein**	**lutein**
Control									
Mean ± SEM	11.05± 2.19 (0.117)**	4.78± 0.62 (0.117)	3.20± 0.30 (0.117)	1.24± 0.10 (0.117)	4.03± 0.17 (0.058)	1.67± 0.38 (0.058)	0.597± 0.06 (0.058)	0.40± 0.01 (0.012)	0.60± 0.10 (0.140)
Treatment Month 12									
Mean± SEM	40.35± 1.30 (0.120)	7.56± 0.71 (0.120)	4.87± 0.70 (0.120)	3.97± 0.55 (0.120)	7.12± 0.22 (0.046)	1.84± 0.58 (0.046)	1.09± 0.06 (0.046)	1.68± 0.91 (0.024)	0.76± 0.08 (0.165)
Month 18									
Mean± SEM	6.20± 0.70 (0.044)	2.34± 0.78 (0.044)	1.45± 0.44 (0.044)	0.66± 0.08 (0.044)	3.64± 1.2 (0.052)	1.39± 0..36 (0.052)	N.D.	0.59± 0.19 (0.005)	1.36± 0.33 (0.042)

* Month 18 values refer to the concentrations measuring in the L-treated primates that were sacrificed 6 months after the end of the supplementation period and month 12 values are those measured in the primates sacrificed immediately following 12 months of supplementation; ** Tissue weights in grams are shown in parentheses.

The data shown in Table 5 indicate a significant increase in the mean concentration of lutein in the retinas of the supplemented animals in comparison to the control group. Figure 11 depicts the changes in mean lutein and zeaxanthin concentrations in the retina and ciliary body of the L-treated primates compared to the controls. The mean lutein

concentration for the retinas of the L-treated group at month 12 (40.35 ± 1.30 ng/tissue) was nearly 4-fold the mean value for the control group (11.05 ± 2.19 ng/tissue) ($p < 0.0001$). At month 18, after 6 months of follow-up without supplementation, the mean lutein level in the retinas of the L-treated primates (6.20 ± 0.70 ng/tissue) was not significantly different ($p = 0.28$) from the mean lutein level of the control group (11.05 ±2.19 ng/tissue). The mean lutein concentration in the ciliary body of the L-treated animals at month 12 (7.12 ± 0.22 ng/tissue), although almost twice that of the control group (4.03 ± 0.17 ng/tissue), was not significantly higher ($p = 0.22$), nor was it significantly higher than the mean level at month 18 ($p = 0.17$). It should be noted that small sample size and inter-individual variability made it difficult to detect smaller differences between groups. The mean concentrations of lutein in the iris was higher in the L-treated group when compared to the controls and the L-treated group at 18-months, but the overall levels were low and variability among animals within the L-treated group was high. Lutein levels in the lens do not appear to have been affected by supplementation.

After 12 months of supplementation, the mean zeaxanthin level in the retina for the L-treated primates was not significantly different ($p = 0.35$) from the control mean or from the mean level at month 18 ($p = 0.10$). Similarly, the mean zeaxanthin level in the ciliary body of the L-treated primates was not significantly different from that of the control group or the mean level measured at month 18 in the L-treated group. No detectable amounts of zeaxanthin were found in the iris and lens tissues of the control and L-treated primates at months 12 or 18.

The mean level of *meso*-zeaxanthin in the retina of the L-treated primates at month 12 was not significantly different ($p = 0.11$) from the control group, but was significantly higher ($p = 0.004$) than the mean level of the L-treated primates after 18 months.

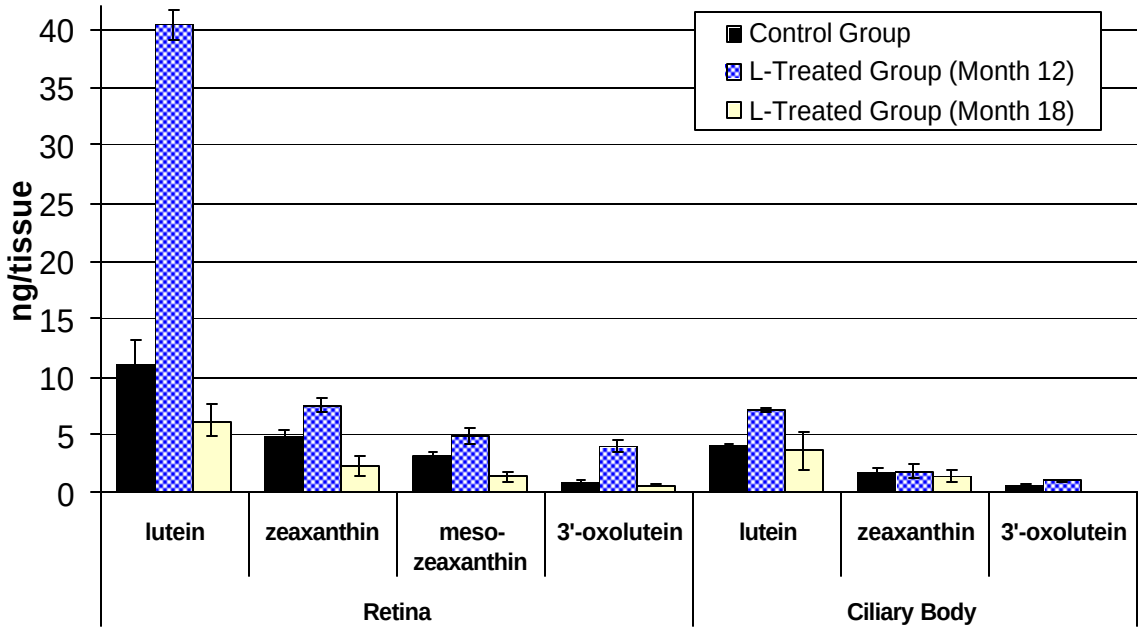


Figure 11. Mean concentrations of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein (ng/tissue) in the retina and ciliary body of the L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) at month 12 (n=2) and month 18 (n=3) compared to the control primates (n= 3). (One control primate was sacrificed at month 12 and 2 were sacrificed at month 18.)

The mean level for the metabolite 3'-oxolutein in the L-treated primates at month 12 was significantly higher ($p = 0.002$) when compared to the control group, and was also significantly higher ($p < 0.001$) than the mean level for the L-treated primates at month 18. This 18 month value, however, was not significantly different from the control mean.

Carotenoid Concentrations in the Major Organs and Tissues of Primates in the Lutein Supplementation Study

The major organs and tissues of one control primate (RQ4079) and 2 L-treated primates (9.34 mg/kg L and 0.66 mg/kg Z daily for 12 months) (RQ4087, RQ4122) were sacrificed at the end of the 12-month supplementation period and were analyzed for carotenoids, their metabolites, and retinol. The data for the control primate in comparison to the mean values for the L-treated group are shown in Table 6. Complete data for each primate are included as Appendix C. Lutein, zeaxanthin, and 3'-oxolutein were detected in all tissues analyzed with the exception of the livers which did not contain any detectable amounts of 3'-oxolutein. Although there was considerable inter-individual variability among the primates in the L-treated group, the mean lutein levels in the primates supplemented with lutein were significantly higher than the mean levels measured in all tissues of the control group. The highest concentrations of carotenoids and retinol were found in the liver of the primates. Another point worth noting is that inconsistencies in the adipose tissue samples analyzed resulted from the varied sources of adipose tissue. Multiple adipose samples were provided for each of the primates that was sacrificed, but the locations from which these adipose tissues were collected were not specified.

Two of the control primates and 3 of the L-treated primates were sacrificed at month 18, following 6 months of observation. The liver, lung, colon, and breast tissues of these primates were analyzed for carotenoids, their metabolites, and retinol. As shown in Table 7, the mean lutein levels observed in the L-treated primates at month 18 were considerably lower than the mean levels observed in the L-treated primates that were sacrificed after 12 months of supplementation. Complete data for each of these primates are tabulated in Appendix D. There was considerable inter-individual variability in the

lutein concentrations in the breast tissue of the primates, making these results difficult to interpret. Mean lutein levels in lung and colon tissues of the L-treated primates at 18 months were closer to the mean levels observed for the control group than they were to the mean levels for the L-treated primates whose tissues were analyzed at month 12. While the mean concentration of lutein in the liver tissues of the L-treated primates was still high at month 18, the value was lower that that of the L-treated primates at month 12.

Table 6. Concentrations (µg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in tissues of one control primate and 2 primates supplemented daily (9.34 mg/kg L and 0.66 mg/kg Z) for 12 months; carotenoids were measured at the end of the 12 month supplementation period.

Tissue	**Primate ID**		**Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues***			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Liver	Control	RQ4079	1.653	0.7751	N.D.	11.331
	Treatment	Mean ± SEM	3.076 ± 0.57	0.918 ± 0.29	N.D.	20.402 ± 8.40
Lung	Control	RQ4079	0.100	0.051	0.006	0.164
	Treatment	Mean ± SEM	0.228 ± 0.02	0.052 ± 0.01	0.024 ± 0.002	0.104 ± 0.044
Colon	Control	RQ4079	0.090	0.060	0.036	0.234
	Treatment	Mean ± SEM	0.231 ± 0.13	0.084 ± 0.05	0.088 ± 0.04	0.144 ± 0.030
Kidney	Control	RQ4079	0.049	0.027	0.005	1.442
	Treatment	Mean ± SEM	0.205±0.07	0.074±0.03	0.035±0.007	1.249±0.026
Breast[a]	Control	Mean ± SEM	0.037 ± 0.017	0.020 ± 0.008	0.009 ± 0.003	0.032 ± 0.021
	Treatment	Mean ± SEM	0.362 ± 0.26	0.098 ± 0.06	0.069 ± 0.05	0.054±0.007
Ovaries[b]	Control	RQ4079	0.026	0.017	0.003	0.033
	Treatment	Mean ± SEM	0.071 ± 0.03	0.019 ± 0.003	0.013 ± 0.008	0.058 ± 0.004
Spleen	Control	RQ4079	0.322	0.138	0.029	0.134
	Treatment	Mean ± SEM	0.620 ± 0.06	0.143 ± 0.02	0.062±0.01	0.125 ± 0.015
Cervix	Control	RQ4079	0.017	0.013	0.002	0.028
	Treatment	Mean ± SEM	0.051 ± 0.01	0.019 ± 0.01	0.011±0.004	0.057 ± 0.037
Adipose	Control	RQ4079	0.163	0.095	0.072	0.147
	Treatment	Mean ± SEM	0.794 ± 0.82	0.187 ± 0.16	0.407	0.133

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers and given as total lutein and zeaxanthin values, respectively; [a] Value for control breast tissue is the mean of the right and left breast tissues for primate 4079; [b] Concentrations of carotenoids and retinol for ovaries are expressed in µg/0.1 g of ovaries rather than µg/g of tissue; the weight of each ovary is between 0.2-0.6 g; N.D., Not detected.

Table 7. Mean concentrations (μg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in tissues of 2 control primates and 3 primates supplemented daily with lutein (9.34 mg/kg L and 0.66 mg/kg Z) at month 18 (12 months supplementation plus 6 months treatment-free).

Tissue	Primate ID		Concentrations (μg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein,and Retinol in Primate Tissues*			
			lutein	zeaxanthin	3'-oxolutein	retinol
Liver	**Control**	Mean ±SEM	0.937 ± 0.44	0.577 ± 0.30	N.D.	35.101 ± 6.79
	Treatment	Mean±SEM	2.006 ± 0.99	0.745 ± 0.19	0.035 ± 0.007	25.461 ± 2.67
Lung	**Control***	Mean±SEM	0.102 ± 0.002	0.059 ± 0.005	0.010 ± 0.001	0.094 ± 0.001
	Treatment*	Mean±SEM	0.136 ± 0.12	0.061 ± 0.022	0.018 ± 0.008	0.177 ± 0.010
Colon	**Control***	Mean±SEM	0.256 ± 0.033	0.144 ± 0.008	0.055 ± 0.016	0.276 ± 0.078
	Treatment*	Mean±SEM	0.199 ± 0.065	0.081 ± 0.027	0.038±0.012	0.217 ± 0.074
Breast	**Control***	Mean±SEM	0.457 ± 0.17	0.289 ± 0.24	0.125 ± 0.033	0.210 ± 0.075
	Treatment*	Mean±SEM	0.347 ± 0.053	0.121 ± 0.029	0.137 ± 0.038	0.084 ± 0.051

* Trace amount of 3'-epilutein was also detected; N.D. = not detected.

Zeaxanthin Supplementation Study: Daily Supplementation of Female Rhesus Macaque Monkeys with Zeaxanthin (10 mg/kg) for 12 Months

Plasma Carotenoid Analysis of Primates in the Zeaxanthin Supplementation Study

The concentrations of zeaxanthin in the plasma of the Z-treated primates at baseline, 6 and 12 months of supplementation, and 6 months post-supplementation (18 months) are shown in Table 8 and depicted in Figure 12. The changes in mean plasma zeaxanthin concentrations of the Z-treated primates compared to those of the control group are depicted in Figure 13.

Table 8. Concentrations (μmol/L) of zeaxanthin in the plasma of 5 primates supplemented daily with zeaxanthin (10 mg/kg) for 12 months at baseline, 6 and 12 months of supplementation, and at 6 months post-supplementation (month 18).

	Total Zeaxanthin Concentration in Plasma (μmol/L)*			
Primate ID	**Baseline**	**6 Months**	**12 Months**	**18 Months**
Control Group **Mean ± SEM**	**0.118 ± 0.009[a]**	**0.069 ± 0.010[a]**	**0.120 ± 0.027[a]**	**0.120 ± 0.026[a]**
Treatment Group				
RQ4120	0.254	0.598	0.840	0.088
RQ4092	0.228	0.760	0.988	0.153
RQ4094	0.172	1.025	1.063	0.150
RQ4146	0.347	0.945	1.147	--[a]
RQ4173	0.150	0.781	0.550	--[a]
Mean ± SEM [b]	**0.230 ± 0.035[a]**	**0.822 ± 0.075[b]**	**0.918 ± 0.104[b]**	**0.130 ± 0.021[a]**

* Total zeaxanthin refers to the combined concentrations of *all-trans*-zeaxanthin and its *cis*-isomers; [a] primate sacrificed after 12 months of supplementation; [b] SEM = standard error of mean; Values with different letters (a, b, c) denote statistical significance across each row and within each column.

For plasma carotenoid analysis the mean baseline values for the Z-treated primates served as their own controls for comparing the mean carotenoid concentrations at other time points. These mean values were also compared to those of the control group. Statistical analysis of the data shown in Table 8 indicates that the mean plasma concentration of zeaxanthin for the primates at baseline (0.230 ± 0.035 μmol/L) was

significantly different than the mean levels at month 6 (0.822 ± 0.075 µmol/L; $p < 0.001$) and at month 12 (0.918 ± 0.104 µmol/L; $p < 0.001$) (see Figures 12 and 13). However, it must be noted that the mean plasma concentration for this group was nearly 2-fold that of the mean plasma zeaxanthin concentration at baseline for the control group (0.118 ± 0.009 µmol/L). The mean plasma zeaxanthin level in the Z-treated primates was not significantly different ($p = 0.38$) between months 6 and 12. This suggests that the plasma concentration of zeaxanthin plateaus at some point between 6 and 12 months of daily supplementation with zeaxanthin. The mean plasma zeaxanthin concentration at month 18 (0.130 ± 0.021 µmol/L) was lower than the baseline value for this group (0.230 ± 0.030 µmol/L) at month 18 (12 months supplementation plus 6 months treatment-free).

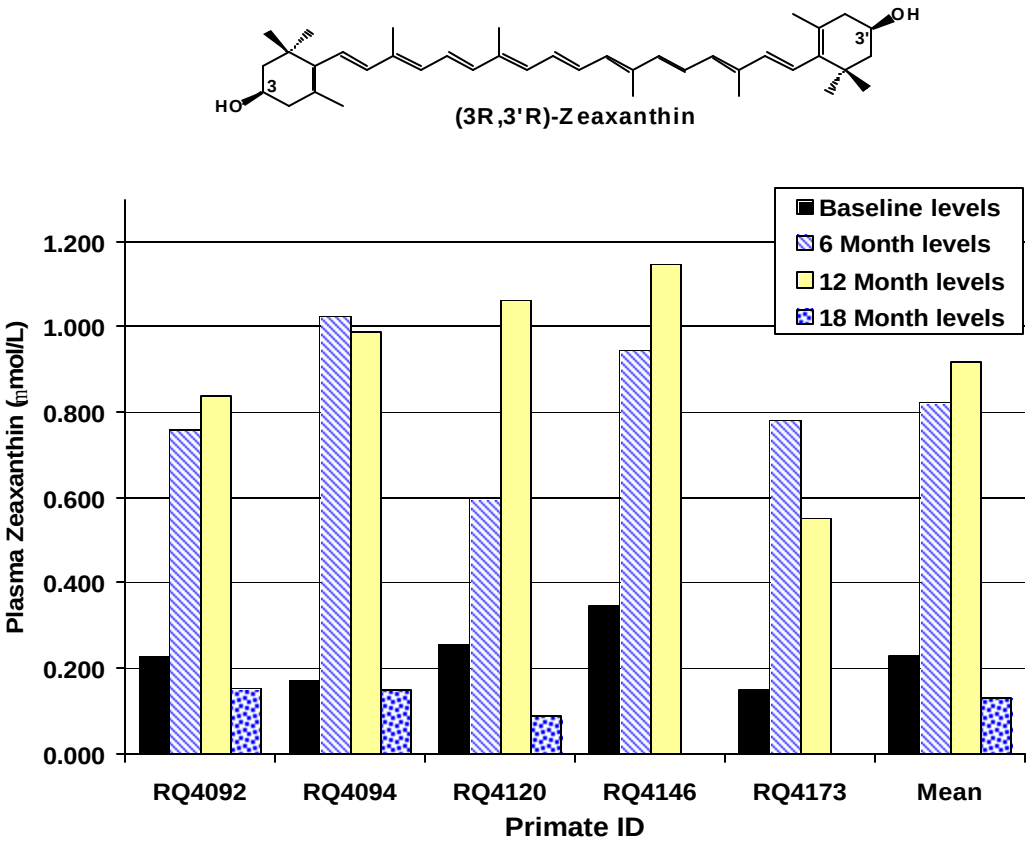


Figure 12. Changes in the plasma concentrations (µmol/L) of zeaxanthin in the Z-treated primates (10 mg zeaxanthin/kg daily for 12 months) at baseline, months 6, 12, and 18.

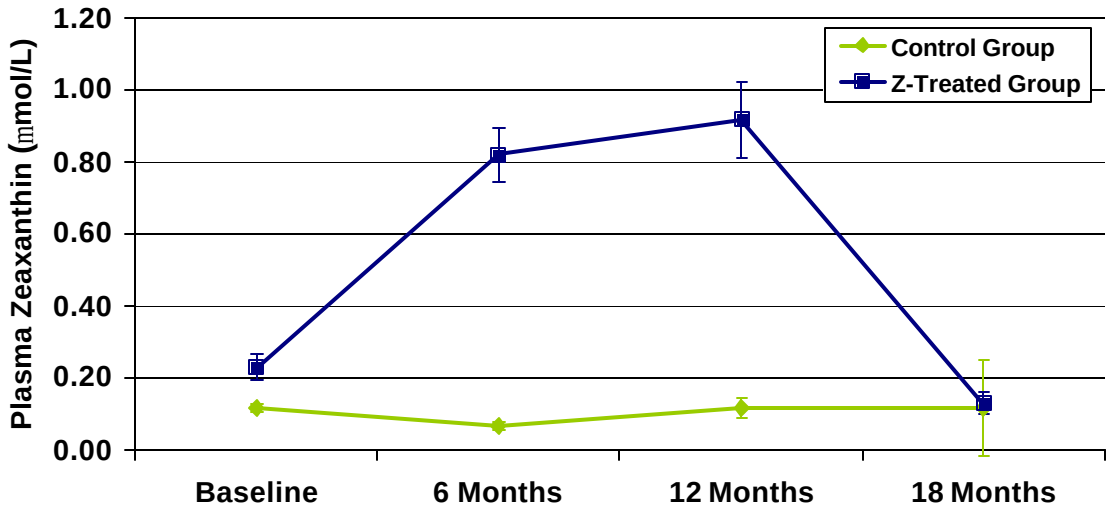


Figure 13. Changes in the mean plasma zeaxanthin concentrations (µmol/L) of the Z-treated primates (10 mg/kg Z daily for 12 months) in comparison with the control primates at baseline, months 6, 12 , and 18.

The mean plasma concentration of lutein of the Z-treated primates at baseline (0.250 ± 0.06 µmol/L) was not significantly different ($p = 0.81$) in comparison with the mean level at month 12 (0.316 ± 0.088 µmol/L) (Figure 14). And, while the mean lutein level did increase between baseline and month 6 (0.416 ± 0.071 µmol/L), this increase was not significant ($p = 0.07$), nor was the decrease observed in the mean plasma lutein concentration between months 6 and 12 ($p = 0.10$). The mean plasma lutein level at month 18 (0.316 ± 0.067 µmol/L) was not significantly different from the mean plasma lutein level at baseline (0.303 ± 0.050 µmol/L) (Figure 14). It must be noted that the zeaxanthin supplements did not contain any lutein.

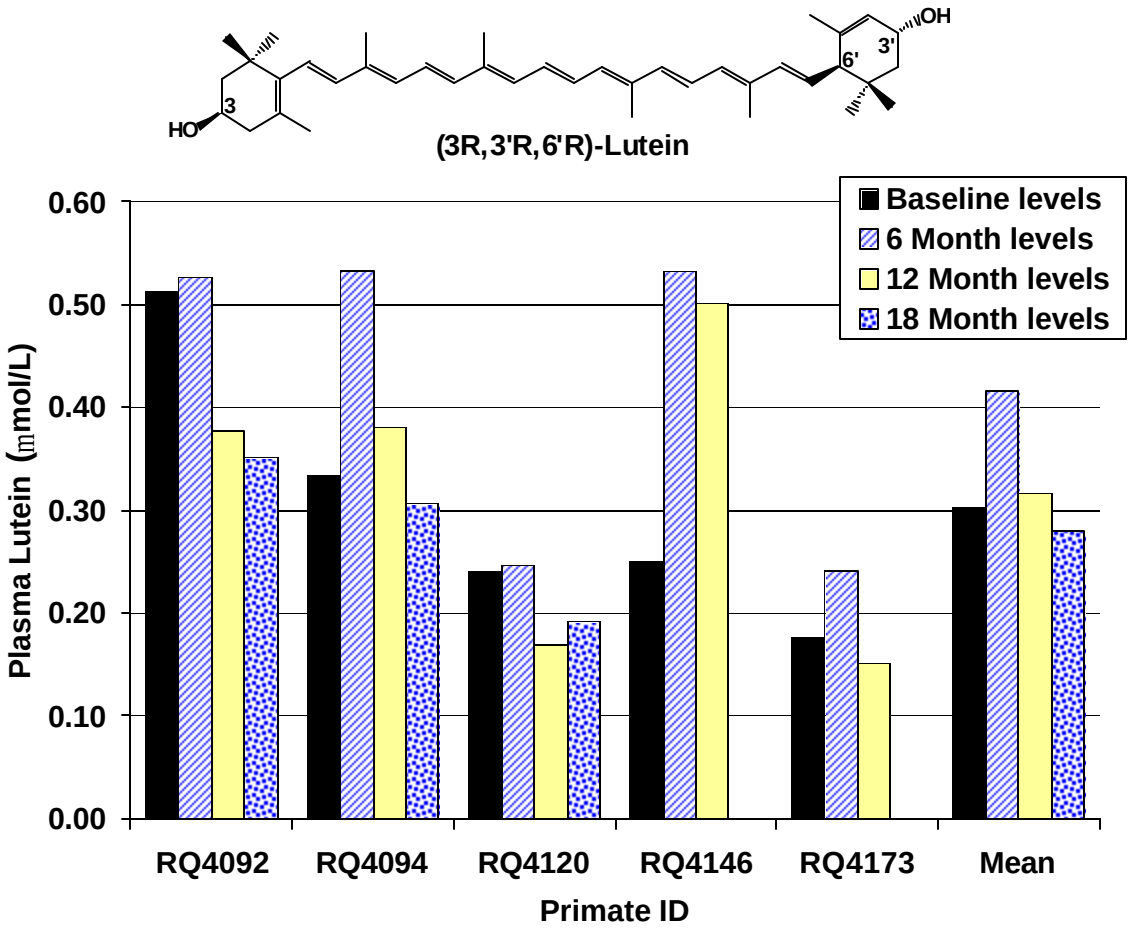


Figure 14. Changes in the plasma concentrations of lutein in the Z-treated primates (10 mg /kg of Z daily for 12 months) at baseline and months 6, 12, and 18.

Plasma Concentrations of Lutein and Zeaxanthin Metabolites in Primates in the Zeaxanthin Supplementation Study

Supplementation of primates with zeaxanthin for 12 months did not seem to alter the plasma concentrations of 3'-epilutein. While trace amounts of 3'-epilutein were detected at baseline, this metabolite was not detected at month 6 and only trace amounts were detected in a couple of the primates at month 12.

The results showed that the mean plasma concentration of 3'-oxolutein at baseline (0.057 ± 0.011 μmol/L) was significantly lower than the mean levels observed at month 6

(0.079 ± 0.011 µmol/L; p = 0.04) and month 12 (0.086 ± 0.011 µmol/L; p = 0.01). However, the mean plasma 3'-oxolutein levels were not significantly different (p = 0.54) between months 6 and 12 of supplementation. These data are listed in Table 9 and are shown in Figure 15. The mean plasma concentration of 3'-oxolutein measured at month 18 (0.041 ± 0.024 µmol/L) decreased below the mean baseline level (0.057 ± 0.007 µmol/L); although it should be noted that the inter-individual variability in plasma concentrations at month 18 was larger than that observed at baseline.

Table 9. Concentrations (µmol/L) of 3'-oxolutein in the plasma of primates supplemented daily with zeaxanthin (10 mg/kg) for 12 months at baseline, months 6, 12, and 18.

	3'-Oxolutein Concentration in Plasma (µmol/L)			
Primate ID	**Baseline**	**6 Months**	**12 Months**	**18 Months**
RQ4120	0.044	0.052	0.074	0.020
RQ4092	0.075	0.064	0.076	0.093
RQ4094	0.052	0.110	0.096	0.041
RQ4146	0.075	0.109	0.132	--[†]
RQ4173	0.040	0.061	0.049	--[†]
Mean ± SEM[‡]	**0.057 ± 0.007[a]**	**0.079 ± 0.026[b]**	**0.086 ± 0.014[b]**	**0.041 ± 0.024[a]**

[†] Primate sacrificed after 12 months of supplementation; [‡] SEM = standard error of mean; Values with different letters (a, b) denote statistical significance between the mean values.

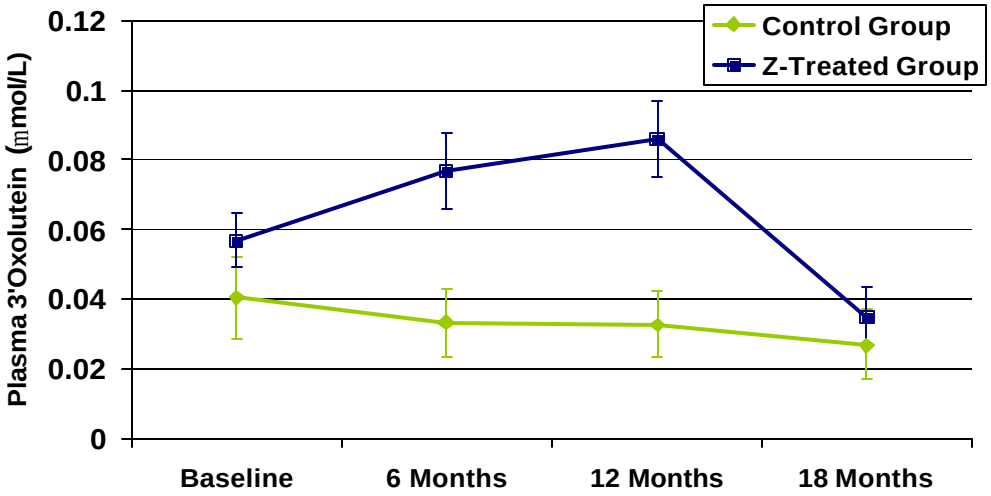


Figure 15. Changes in the mean plasma concentrations (µmol/L) of 3'-oxolutein in the Z-treated primates at baseline, months 6, 12, and 18.

Concentrations of Carotenoids in the Ocular Tissues of Primates in the Zeaxanthin Supplementation Study

After 12 months of daily supplementation with zeaxanthin (10 mg/kg), 2 primates were sacrificed and the ocular tissues (retina, ciliary body, iris, lens) from one eye of each animal were extracted and analyzed for zeaxanthin, lutein, their metabolites, and retinol. Six months post-supplementation, the remaining 3 primates were sacrificed, and in most cases, the tissues from both the right and left eyes of each animal were analyzed. The mean concentrations of carotenoids in the ocular tissues of the Z-treated group at month 12 and month 18 compared to the control group are shown in Table 11. Complete data for carotenoid and metabolite concentrations of the ocular tissues of each of the Z-treated primates are attached as Appendix E. The mean concentrations of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein in the ocular tissues of the Z-treated primates at month 12 were compared to the mean values for the 3 control primates (RQ4079, RQ4107, and RQ4142).

The data depicted in Figure 16 clearly indicate increases in the mean concentrations of zeaxanthin in the retina and ciliary body of the Z-treated primates at month 12 in comparison to the mean levels of the control primates as well as the Z-treated primates at month 18. The mean concentration of zeaxanthin in the retina of the Z-treated primates at month 12 (20.43 ± 3.88 ng/tissue) was greater than 4-fold that of the mean value of the control primates (4.78 ± 0.62 ng/tissue) ($p < 0.001$). The month 12 value for the Z-treated primates was also significantly higher ($p < 0.001$) than that of the Z-treated primates at month 18 (6.85 ± 0.82 ng/tissue) which was not significantly different ($p = 0.89$) from that of the control. It should be noted that the mean retina weight for the control primates was 0.117 g and that the mean retina weight of the Z-treated

primates sacrificed at month 18 was only 0.041 g The mean concentration for total zeaxanthin in the ciliary body of the Z-treated primates at month 12 (12.85 ± 4.28 ng/tissue) was significantly higher ($p < 0.001$) than that of the control primates (1.67 ± 0.38 ng/tissue), and was also significantly higher ($p = 0.003$) than the mean level for the Z-treated primates at month 18 (3.53 ± 0.62 ng/tissue). While there was no zeaxanthin detected in the iris tissues of either the control primates or the Z-treated primates at month 18, a small amount of this carotenoid was detected in the Z-treated group at month 12. The amounts of zeaxanthin detected in the lens were also small, yet the mean level at month 12 was almost twice that of the control group. No zeaxanthin was detected in the lens of the Z-treated primates at month 18.

Table 10. Mean concentrations (ng/tissue) of lutein, zeaxanthin, *meso*-zeaxanthin and 3'-oxolutein in ocular tissues of the Z-treated primates at month 12 (n=2) and at month 18 (n=3) compared to the mean levels of the control group (n=3).

Primate Group	Concentrations (ng/tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates Ocular Tissues (Tissue Weight, g)*								
	Retina				Ciliary Body			Iris	Lens
	lutein	zea-xanthin	*meso*-zea-xanthin	3'-oxo-lutein	lutein	zea-xanthin	3'-oxo-lutein	lutein/ zeaxanthin	lutein/ zeaxanthin
Control									
Mean ± SEM	11.05± 2.19 (0.117)	4.78± 0.62 (0.117)	3.20± 0.30 (0.117)	0.82± 0.10 (0.117)	4.03± 0.17 (0.058)	1.67± 0.38 (0.058)	0.60± 0.06 (0.058)	0.26±.01/ N.D. (0.012)	0.60±.10/ 0.18±..05 (0.140)
Z-Treated									
12 months									
Mean ± SEM	19.15± 9.07 (0.105)	20.43± 3.88 (0.105)	7.20± 1.37 (0.105)	3.87± 0.10 (0.105)	4.97± 2.55 (0.053)	12.85± 4.28 (0.053)	1.34± 0.50 (0.053)	0.26±.05/ 0.30±..01 (0.028)	0.34±.01/ 0.37±..05 (0.163)
18 months									
Mean ± SEM	7.842± 1.03 (0.041)	3.93± 0.66 (0.041)	1.78± 0.26 (0.041)	0.58± 0.27 (0.041)	7.18± 1.04 (0.095)	3.53± 0.62 (0.095)	0.95± 0.19 (0.095)	0.65±..01/ N.D. (0.013)	0.63± .01/ N.D. (0.169)

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers and reported as total lutein and total zeaxanthin, respectively; N.D., Not detected; SEM = standard error of the mean.

The mean concentration of lutein in the retina of the Z-treated animals at month 12 was nearly double that of the control primates, but there was a large inter-individual variability, and thus, this difference was not statistically significant ($p = 0.12$). Interestingly, the mean concentration of lutein in the retina of the Z-treated primates at month 12 was significantly higher ($p = 0.04$) than that of the Z-treated primates at month 18. However, the mean concentration of lutein in the retina of the Z-treated animals at the month 18 was not significantly different ($p = 0.47$) than that of the control. The mean lutein levels in the ciliary body were higher in the Z-treated primates than the control at both months 12 and 18, but there was a high degree of inter-animal variability associated with the levels of lutein in this tissue and consequently, the differences were not statistically significant.

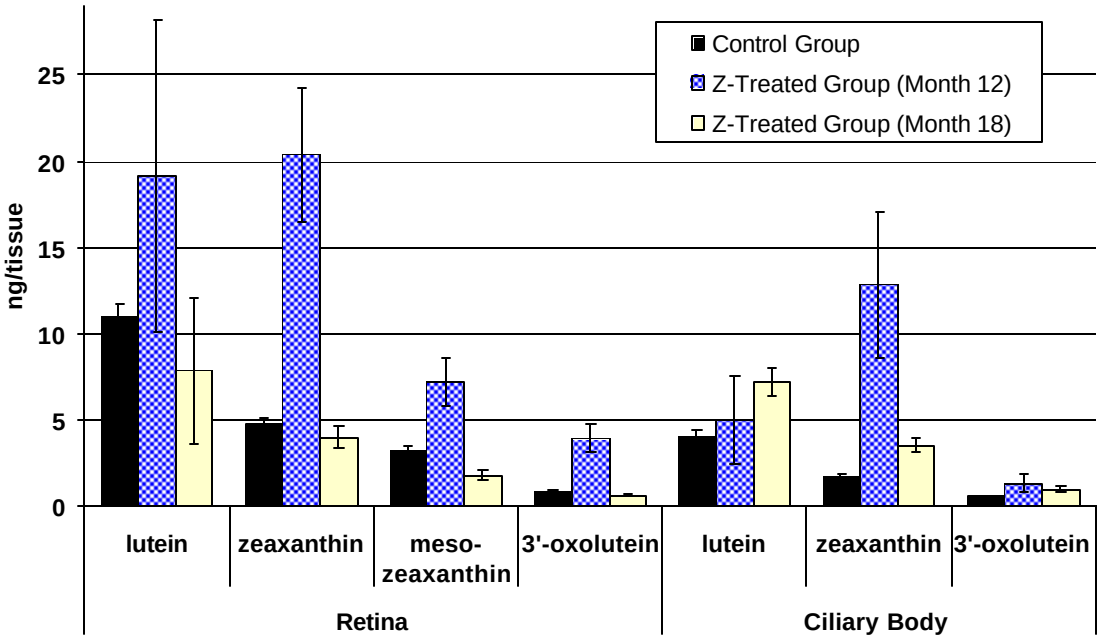


Figure 16. Mean concentrations (ng/tissue) of lutein, zeaxanthin, and their metabolites in the retina and ciliary body of the Z-treated primates at months 12 (n=2) and 18 (n=3) in comparison to the control primates (n=3). (One control primate was sacrificed at month 12 and 2 were sacrificed at month 18.)

The mean concentration of *meso*-zeaxanthin in the retinas of the Z-treated primates at month 12 was significantly higher ($p = 0.001$) than that of the control primates, and that of the Z-treated primates at month 18 ($p < 0.001$).

The mean concentration of 3'-oxolutein in the retina of the Z-treated primates at month 12 was significantly higher than that of the control group ($p = 0.002$) and the Z-treated primates at month 18 ($p < 0.001$). However, the mean level of 3'-oxolutein in the Z-treated group at month 18 was not significantly different from the control mean ($p = 0.52$).

The mean concentration of 3'-oxolutein in the ciliary body of the Z-treated primates had increased by month 12 of supplementation, and levels of this metabolite then decreased during the post-supplementation period (Figure 16).

Carotenoid Concentrations in the Major Organs and Tissues of Primates in the Zeaxanthin Supplementation Study

Other major organs and tissues of the sacrificed animals were also analyzed for carotenoids, their metabolites, and retinol. The organs and tissues analyzed in the primates sacrificed at month 12 were: liver, lung, colon, kidney, breast, ovaries, spleen, cervix, and adipose. The mean concentrations of lutein, zeaxanthin, their metabolites, and retinol for the 2 Z-treated primates sacrificed at month 12 were compared to that of the control primate (RQ4079). These data are listed in Table 11 and the complete data for each primate are attached as Appendix F. In all cases, the concentrations of zeaxanthin in the tissues and organs of the Z-treated primates were significantly higher than the values obtained for the control primate. As expected, the liver tissues accumulated the highest concentrations of carotenoids and retinol.

Table 11. Mean concentrations (µg/g tissues) of lutein, zeaxanthin, 3'-oxolutein, and retinol in tissues of two Z-treated primates (10 mg/kg Z daily for 12 months) in comparison with those of the control primate (RQ4079).

Tissue	Primate Group	Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues*			
		lutein	zeaxanthin	3'-oxolutein	retinol
Liver	Control [a]	1.653	0.775	N.D.	11.331
	Treatment [b]	1.732 ± 0.991	3.890 ± 0.828	0.147 ± 0.044	11.116 ± 0.623
Lung	Control	0.100	0.051	0.006	0.164
	Treatment	0.052 ± 0.00	0.166 ± 0.051	0.013 ± 0.009	0.082± 0.062
Colon	Control	0.090	0.060	0.036	0.234
	Treatment	0.288 ± 0.215	1.085 ± 1.468	0.023 ± 0.00	0.036 ± 0.003
Breast	Control	0.037 ± 0.017	0.020 ± 0.008	0.009 ± 0.003	0.042 ± 0.019
	Treatment	0.081± 0.025	0.365 ± 0.074	0.044 ± 0.012	0.042 ± 0.005
Kidney	Control	0.049	0.027	0.005	1.442
	Treatment	0.055 ± 0.029	0.125 ± 0.036	0.012 ± 0.004	1.149 ± 0.056
Ovaries**	Control	0.026	0.017	0.003	0.033
	Treatment	0.013 ± 0.001	0.041 ± 0.014	0.002 ± 0.001	0.016 ± 0.004
Spleen	Control	0.322	0.138	0.029	0.134
	Treatment	0.169 ± 0.109	0.373 ± 0.27	0.022 ± 0.004	0.045 ± 0.004
Cervix	Control	0.017	0.013	0.002	0.028
	Treatment	0.043 ± 0.028	0.082 ± 0.032	0.009 ± 0.004	0.037 ± 0.003
Adipose	Control	0.163	0.095	0.072	0.147
	Treatment	0.040 ± 0.026	0.125 ± 0.048	0.016 ± 0.010	0.017 ± 0.003

* Concentrations of *cis*-isomers of lutein and zeaxanthin were combined with their corresponding *all-trans* isomers; ** Concentrations of carotenoids and retinol for ovaries are expressed in µg/0.1 g of tissue rather than µg/g of tissue; the weight of each ovary was between 0.2-0.3 g; [a] Control values are the concentrations for primate RQ4079; [b] Treatment values are the mean ± SEM for primates RQ4146 and RQ4173; N.D., Not detected.

After the 6-month post-supplementation (observation) period, the remaining 3 primates in the Zeaxanthin Supplementation Study were sacrificed and the following organs and tissues were analyzed for lutein, zeaxanthin, their metabolites, and retinol: liver, lung, colon, and breast. These concentrations were compared to those of the 2 control primates sacrificed at month 18. The mean tissue concentrations of carotenoids for the Z-treated and control primates are shown in Table 12; the complete set of data for

each primate is attached as Appendix G. The mean level of zeaxanthin in each of these tissues at month 18 was significantly lower than the mean level observed in the corresponding tissues of the Z-treated primates at month 12. Although there was a large variability among the primates, the levels of zeaxanthin in these tissues at month 18 appear to be in the same range of concentrations as those of the control primates.

Table 12. Mean concentrations (µg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol six months post-supplementation in the tissues of three Z-treated primates in comparison to those of the control primates.

Tissue	**Primate ID**		**Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues** *			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Liver						
	Control	Mean±SEM	0.937 ± 0.537	0.577 ± 0.296	N.D.	35.101 ± 6.79
	Treatment	Mean±SEM	1.610 ± 0.133	0.746 ± 0.149	N.D.	147.58 ±31.40
Lung						
	Control	Mean±SEM	0.102 ± 0.065	0.059 ± 0.005	0.010 ± 0.00	0.094 ± 0.001
	Treatment	Mean±SEM	0.152 ± 0.010	0.078 ± 0.005	0.013 ± 0.002	0.191 ± 0.035
Colon						
	Control	Mean±SEM	0.256 ± 0.033	0.144 ± 0.121	0.055 ± 0.016	0.276 ± 0.078
	Treatment	Mean±SEM	0.266 ± 0.072	0.135 ± 0.050	0.026 ± 0.007	0.237 ± 0.051
Breast						
	Control	Mean±SEM	0.457 ± 0.167	0.289 ± 0.129	0.125 ± 0.033	0.210 ± 0.075
	Treatment	Mean±SEM	0.227 ± 0.087	0.178 ± 0.077	0.064 ± 0.022	0.119 ± 0.083

* Concentrations of *cis*-isomers of lutein and zeaxanthin were combined with their corresponding *all-trans* isomers; N.D., Not detected.

Lutein/Zeaxanthin (L/Z) Supplementation Study: Daily Supplementation of Female Rhesus Macaque Monkeys with a 1:1 Combination of Lutein and Zeaxanthin (0.5 mg/kg of each) for 12 Months

Plasma Carotenoid Analysis of Primates in the Lutein/Zeaxanthin Supplementation Study

For the L/Z Supplementation Study, 5 primates were supplemented daily with a combination of lutein and zeaxanthin each at a dose of 0.5 mg/kg for 12 months. At the end of the 12-month supplementation period, all of the primates were sacrificed. As in the 2 previously described studies, primate plasma samples were analyzed at baseline, 6 months, and 12 months for lutein, zeaxanthin, their metabolites, and retinol. These data are summarized in Table 13 and Figure 17.

Table 13. Concentrations (µmol/L) of carotenoids and their metabolites in the plasma of 5 L/Z-treated primates (0.5mg/kg L & 0.5mg/kg Z daily for 12 months) at baseline and months 6 and 12.

Primate ID		**Concentration in Plasma (µmol/L)***			
		lutein	**zeaxanthin**	**3'-epilutein**	**3'-oxolutein**
Baseline	RQ4086	0.320	0.098	0.013	0.044
	RQ4154	0.243	0.153	0.033	0.044
	RQ4118	0.214	0.090	0.015	0.038
	RQ4126	0.182	0.062	0.015	0.016
	RQ4104	0.332	0.147	0.019	0.031
	Mean ± SEM†	**0.258 ± 0.029[a]**	**0.111 ± 0.017[a]**	**0.019 ± 0.004[a]**	**0.034 ± 0.005[a]**
6 Months	RQ4086	0.689	0.384	0.015	0.082
	RQ4154	0.614	0.409	0.066	0.062
	RQ4118	0.621	0.282	0.028	0.071
	RQ4126	0.756	0.397	0.031	0.061
	RQ4104	0.390	0.200	0.011	0.033
	Mean ± SEM†	**0.614 ± 0.061[b]**	**0.334 ± 0.040[b]**	**0.030 ± 0.010[a]**	**0.062 ± 0.008[b]**
12 Months	RQ4086	0.408	0.188	0.021	0.030
	RQ4154	0.210	0.081	0.026	0.030
	RQ4118	0.209	0.106	0.021	0.026
	RQ4126	0.236	0.108	0.029	0.038
	RQ4104	0.273	0.199	0.008	0.026
	Mean ± SEM†	**0.278 ± 0.037[a]**	**0.1356 ± 0.0238[a]**	**0.021 ± 0.004[a]**	**0.030 ± 0.002[a]**

* Concentrations of *cis*-isomers of lutein and zeaxanthin were combined with their corresponding *all-trans* isomers; † SEM = standard error of mean; Values with different letters (a, b) denote statistical significance within each column.

From baseline to month 6 of supplementation there was a 2-fold increase in the mean plasma level of lutein among the L/Z-treated primates. The mean plasma lutein level increased significantly ($p < 0.001$) between baseline (0.258 ± 0.029 µmol/L) and 6 months (0.614 ± 0.061 µmol/L). However, despite the fact that supplementation with lutein and zeaxanthin continued between months 6 and 12, there was no significant difference ($p = 0.89$) between the mean plasma levels of lutein at baseline (0.258 ± 0.029 µmol/L) and month 12 (0.267 ± 0.037 µmol/L). The mean plasma concentrations of lutein in the L/Z-treated primates in comparison with the controls are shown in Figure 18. These values indicate that in primates supplemented daily with lutein (0.5 mg/kg) and zeaxanthin (0.5 mg/kg), the plasma concentration of lutein is increased within 6 months of supplementation, but then returns to the baseline level between months 6 and 12.

(3R,3'R,6'R)-Lutein

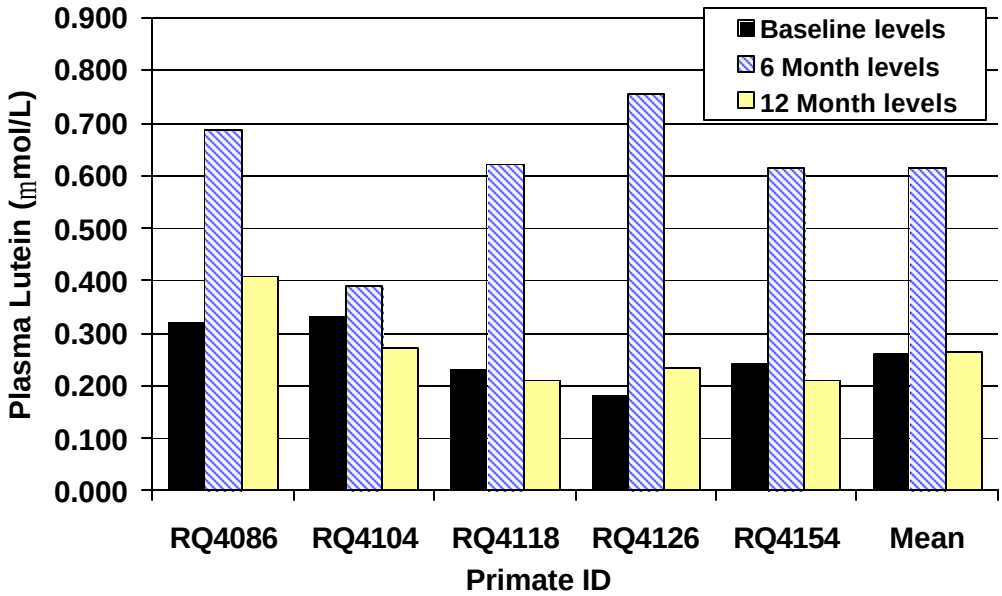


Figure 17. Changes in the plasma lutein concentrations (µmol/L) of the L/Z-treated primates (0.5mg/kg L and 0.5mg/kg Z daily for 12 months) at baseline and months 6 and 12.

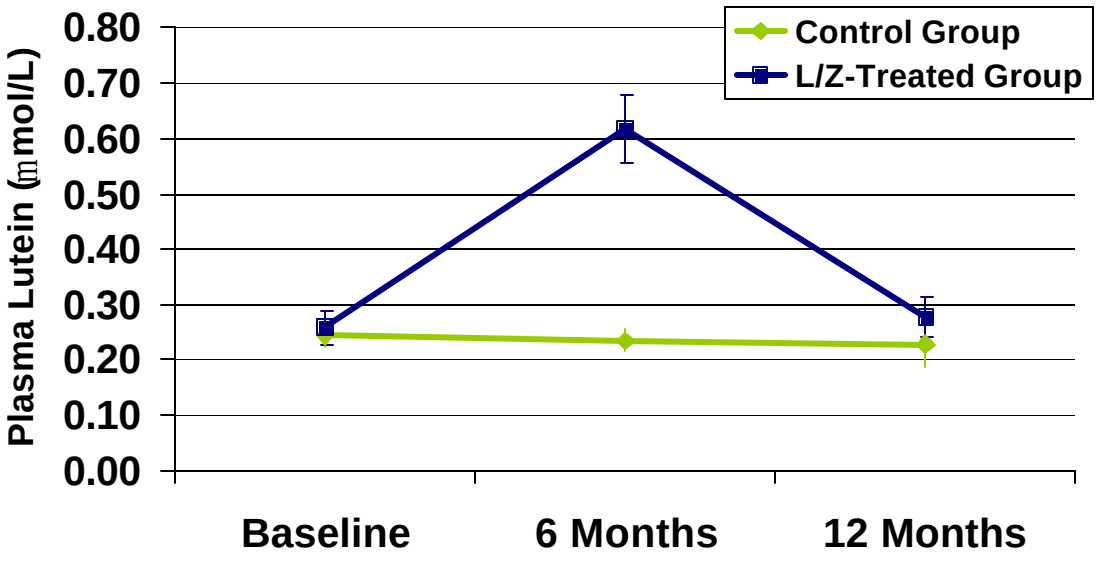
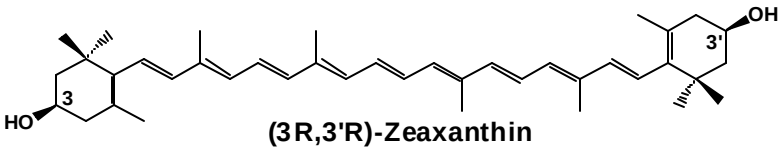


Figure 18. Changes in the mean plasma concentrations of lutein in the L/Z-treated primates (n = 5) (0.5mg/kg L and 0.5mg/kg Z daily for 12 months) and the control primates (n = 3).

A 3-fold increase in the mean plasma zeaxanthin concentration was observed between baseline and month 6 of supplementation. Plasma zeaxanthin concentrations for each primate are depicted in Figure 19 and the mean levels for the L/Z-treated primates compared to those of the control primates are shown in Figure 20. The mean plasma zeaxanthin level at month 6 (0.334 ± 0.040 μmol/L) was significantly higher ($p < 0.001$) than the mean level measured at baseline (0.111 ± 0.017 μmol/L). Interestingly, as seen in the mean lutein levels of the L/Z-treated primates, the mean plasma concentration of zeaxanthin at month 12 of supplementation (0.136 ± 0.024 μmol/L) was not significantly different ($p = 0.48$) than that at baseline (0.111 ± 0.017 μmol/L).



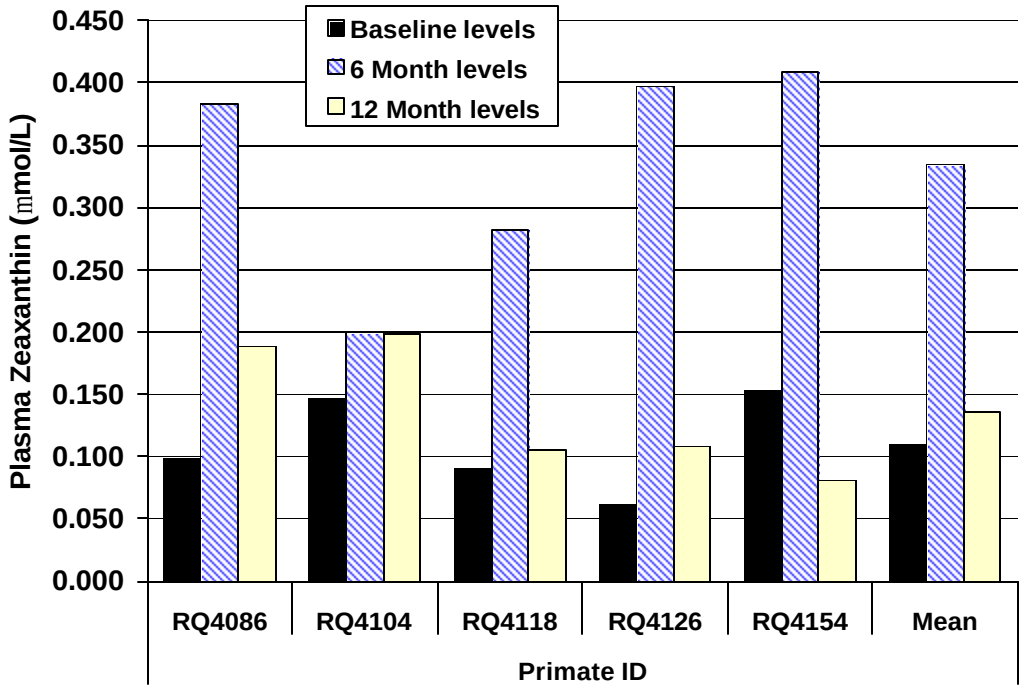


Figure 19. Changes in the mean plasma concentrations of zeaxanthin (µmol/L) in the L/Z-treated primates at baseline and months 6 and 12.

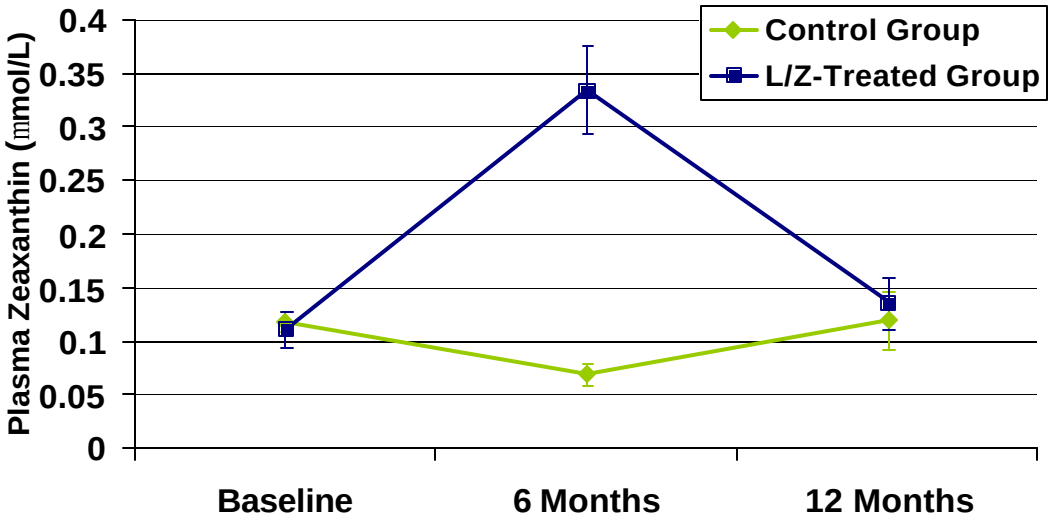


Figure 20. Changes in the me an plasma zeaxanthin concentrations (µmol/L) of the L/Z-treated primates in comparison to the control group at baseline and months 6 and 12.

Plasma Concentrations of Lutein and Zeaxanthin Metabolites in Primates in the Lutein/Zeaxanthin Supplementation Study

There were no significant changes in the mean plasma concentrations of lutein and zeaxanthin metabolites between baseline and month 12 of supplementation. Plasma concentrations of 3'-oxolutein and 3'-epilutein for each of the L/Z-treated primates are tabulated in Table 13. Plasma levels of 3'-oxolutein and 3'-epilutein increased between baseline and month 6, but decreased thereafter and returned to nearly their baseline levels at month 12. A significant increase ($p = 0.003$) in the mean plasma concentration of 3'-oxolutein occurred between baseline (0.034 ± 0.005 µmol/L) and month 6 (0.062 ± 0.008 µmol/L), yet there was no significant difference ($p = 0.69$) between the mean level of this metabolite at month 12 (0.030 ± 0.002 µmol/L) and baseline (0.034 ± 0.005 µmol/L). The mean 3'-oxolutein plasma levels for the L/Z-treated primates are depicted in Figure 21. Although the mean plasma concentration of 3'-epilutein increased by 1.5-fold between baseline and month 6, there were no statistically significant differences between the mean levels at baseline and month 6 ($p = 0.22$) or month 12 ($p = 0.60$).

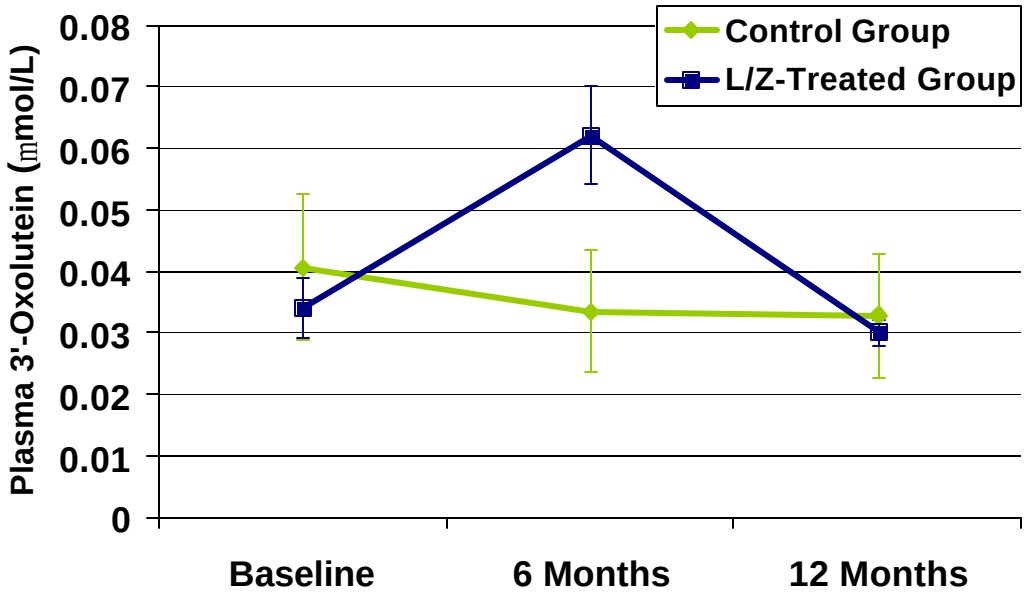


Figure 21. Changes in the mean plasma concentrations of 3'-oxolutein in the L/Z-treated primates in comparison to the control primates at baseline and months 6 and 12.

Concentrations of Carotenoids in the Ocular Tissues of Primates in the L/Z Supplementation Study

The ocular tissues (retina, ciliary body, iris, lens) from both right and left eyes of the L/Z-treated primates were extracted and analyzed for lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein at the end of the 12-month supplementation period. The mean values for lutein, zeaxanthin, and their metabolites for the right and left eyes were used except for several instances in which the tissues from both right and left eyes of a primate were not available; these cases are noted in Appendix H which includes the complete data for the ocular tissues of the L/Z-treated primates. These data are listed in Table 14 and are also shown in Figure 22.

Table 14. Mean concentrations (ng/tissue) of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein in ocular tissues of L/Z-treated primates at month 12 compared to those of the control primates.*

Primate ID	Concentrations (ng/tissue) of Lutein, Zeaxanthin, *meso*-Zeaxanthin, and 3'-Oxolutein in Primates' Ocular Tissues (Tissue Weight, g)								
	Retina				Ciliary Body			Iris	Lens
	lutein	zea-xanthin	*meso*-zea-xanthin	3'-oxo-lutein	lutein	zea-xanthin	3'-oxo-lutein	lutein/ zea-xanthin	lutein/ zea-xanthin
Control									
Mean ± SEM	11.05± 2.19 (0.117)	4.78± 0.62 (0.117)	3.20± 0.30 (0.117)	1.22± 0.096 (0.117)	4.03± 0.17 (0.058)	1.64± 0.38 (0.058)	0.60 ± 0.14 (0.058)	0.26±0.1/ N.D. (0.014)	0.60±0.1/ 0.18±0.2 (0.14)
Treatment									
Mean ± SEM	11.50± 2.12 (0.108)	5.660± 2.02 (0.108)	1.75± 0.54 (0.108)	2.37± 0.33 (0.108)	4.89± 1.66 (0.060)	3.92± 1.17 (0.060	0.80± 0.26 (0.060)	0.77±.18/ 0.52±.15 (0.010)	0.56±.07/ 0.39±.06 (0.107)

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; N.D., Not detected.

The data for the ocular tissues for the L/Z-treated primates show that there were no clear increases in the concentrations of lutein or zeaxanthin in the retina. There were no statistically significant differences in the concentrations of lutein, zeaxanthin, *meso*-zeaxanthin, or 3'-oxolutein in the retina of the primates supplemented daily with 0.5 mg/kg of lutein plus 0.5 mg/kg of zeaxanthin at month 12 when compared to the control group. When examining the total levels of lutein and zeaxanthin, the mean levels of combined total lutein and zeaxanthin in the retina are nearly equal in the control group (19.03 µg/retina) and the L/Z-treated group (18.91 µg/retina). Therefore, the concentrations of lutein and zeaxanthin do not appear to have increased after 12 months of supplementation with the dose of 0.5 mg/kg of each of these carotenoids.

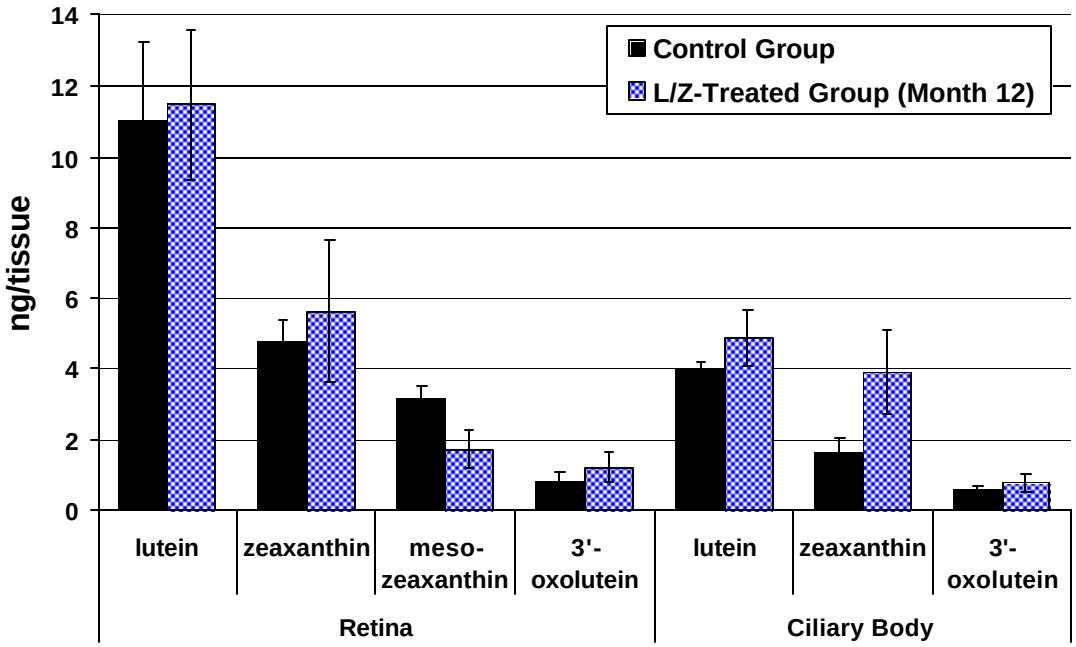


Figure 22. Mean concentrations of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein (ng/tissue) in the retina and ciliary body of the L/Z-treated primates at month 12 (n=5) in comparison with the control primates (n=3). (One control primate was sacrificed at month 12 and 2 were sacrificed at month 18.)

The mean concentrations of lutein, zeaxanthin, and 3'-oxolutein in the ciliary bodies were not significantly higher in the L/Z-treated group at month 12 when compared to those of the control group. The mean concentration of zeaxanthin in the ciliary body of the L/Z-treated primates was greater than 2-fold that of the control group, but it should be noted that there was a high degree of inter-individual variability with regard to these values.

The mean concentration of lutein in the iris of the L/Z-treated primates was almost 3-fold that of the controls. Additionally, while no zeaxanthin was detected in the iris of the control primates, trace amounts were detected in the iris of the L/Z-treated primates. Only low levels of lutein and zeaxanthin were detected in the lens. However, supplementation of the primates with lutein and zeaxanthin resulted in a 2-fold increase in the mean

concentration of zeaxanthin in the lens when compared to that of the control group. Meanwhile, the mean level of lutein did not appear to be affected by supplementation.

Carotenoid Concentrations in the Major Organs and Tissues of Primates in the Lutein/Zeaxanthin Supplementation Study

At the end of the 12-month supplementation period, the major organs and tissues of the 5 L/Z-treated primates were extracted and analyzed for lutein, zeaxanthin, their metabolites, and retinol. The mean concentrations of these carotenoids in this group were compared to those measured in the control primate (RQ4079) (Table 15). Complete data for organs and tissues of each L/Z-treated primate compared to the control primate are presented in Appendix I.

In the colon, kidney, breast, ovary, cervix, and adipose tissues, the mean lutein and zeaxanthin levels were considerably higher in the L/Z-treated primates than in the control primate. For instance, the mean lutein and zeaxanthin concentrations in the colon tissues of the L/Z-treated group were more than 6-fold higher than the concentrations measured for the control animal. Interestingly, the mean levels of lutein and zeaxanthin were higher in the liver tissue of the control primate in comparison to the mean levels observed in the L/Z-treated group. The lutein concentration in the liver of the control primate (1.65 µg/g) was more than 2-fold the mean concentration of the L/Z-treated group (0.72 µg/g). Meanwhile, the zeaxanthin concentration in the liver was almost 2-fold higher in the control primate in comparison to the mean level of the L/Z-treated group.

Table 15. Mean concentrations (µg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in the tissues of the L/Z-treated primates (n=5) compared to control primate RQ4079.

Tissue	Primate Group	Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues*			
		lutein	zeaxanthin	3'-oxolutein	retinol
Liver	Control[a]	1.653	0.755	N.D.	0.113
	Treatment[b]	0.719 ± 0.12	0.479 ± 0.09	N.D.	5.781 ± 3.11
Lung	Control	0.100	0.051	0.006	0.164
	Treatment	0.088 ± 0.02	0.067 ± 0.02	0.014 ± 0.03	0.072 ± 0.006
Colon	Control	0.090	0.060	0.036	0.234
	Treatment	0.550 ± 0.19	0.376 ± 0.16	0.060 ± 0.02	0.383 ± 0.09
Kidney	Control	0.049	0.027	0.005	1.442
	Treatment	0.241 ± 0.13	0.144 ± 0.06	0.028 ± 0.01	1.467 ± 0.13
Breast	Control	0.037	0.020	0.009	0.032
	Treatment	0.440 ± 0.09	0.318 ± 0.07	0.068 ± 0.02	0.501 ± 0.14
Ovaries	Control	0.026	0.017	0.003	0.033
	Treatment	0.478 ± 0.25	0.326 ± 0.23	0.089 ± 0.05	1.336 ± 0.79
Spleen	Control	0.322	0.138	0.029	0.134
	Treatment	0.418 ± 0.06	0.341 ± 0.09	0.085 ± 0.02	0.266 ± 0.025
Cervix	Control	0.017	0.013	0.002	0.028
	Treatment	0.052 ± 0.01	0.032 ± 0.01	0.010 ± 0.003	0.100 ± 0.14
Adipose	Control	0.163	0.095	0.072	0.147
	Treatment	0.638 ± 0.34	0.403 ± 0.26	N.D.	7.661 ± 5.01

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; [a] Value for the control primate RQ4079; [b] Mean ± standard error of the mean (SEM) for L/Z-treated primates; N.D., Not detected.

Safety of High Supplemental Doses (~10mg/kg) of Lutein or Zeaxanthin

Toxicology assays for this study were conducted by other co-investigators of this study. Therefore only a brief summary of these results is included in this thesis.

Fundus Photography and Retina Histopathology

The results from fundus photography and histopathology of retina revealed no abnormalities in any of the animals supplemented daily with a high dose of lutein or zeaxanthin for 12 months. The retinal pigment epithelium (RPE) of all monkeys appeared normal and showed a normal distribution of melanosomes and lipofuscin granules. Bruch's membranes were normal and free of deposits. The choroids and their cellular components were also normal. In addition, there are no signs of inflammation or abnormal numbers of circulating leukocytes in retinal or choroidal blood vessels. The refractile elements seen in gross examination were not evident in histologic sections. Therefore supplementation with lutein or zeaxanthin at high doses would not be expected to cause ocular toxicity.

Urinary Creatinine and Total Protein

The results of studies on total urinary protein excretion showed no statistically significant alterations in protein excretion patterns based upon per milligram of creatinine excreted. Overall, these data suggest that the various lutein and zeaxanthin treatments did not produce any clinical renal damage to the monkeys. The statistically non-significant increase in the urinary protein excretion per mg of creatinine that was observed in the L/Z-treated group was undoubtedly a reflection of the lower urinary creatinine values seen in this group in comparison to the other treatment groups. This is most likely the result of a more dilute urine samples in some of these animals but general maintenance of the protein/creatinine ratio.

DISCUSSION

The study described in this thesis was undertaken in order to determine the effects of high-dose dietary supplementation with lutein and zeaxanthin, and their 1:1 combination on the deposition of these carotenoids and their metabolites in the plasma, ocular tissues, and other organs and major tissues in female Rhesus Macaque primates. To date, several lutein and zeaxanthin supplementation studies in various species of primates have been conducted (Snodderly et al., 1990; Snodderly et al., 1997; Leung et al., 2001; Neuringer et al., 2004). The results discussed in this thesis were part of a larger study that involved ocular examinations including fundus photography and multifocal electroretinograms (ERGs), retina histopathology, and the measurement of various biomarkers associated with toxicity. Thus, this study aimed to explore the efficacy of high-dose lutein and zeaxanthin supplementation as a potential treatment in the prevention and/or attenuation of AMD and to investigate the safety of such treatments. Prior to this study no data were available concerning the potential toxicity from high (pharmaceutical) doses of lutein and zeaxanthin such as those selected for the L-treated and the Z-treated primates. The dose of approximately 10 mg/kg/day that was chosen for lutein and zeaxanthin was approximately 60 times the highest dose selected for a recent clinical trial in older adults with and without AMD (Moura et al., 2004), and 5 times higher than the dose selected for the primates in the study by Neuringer et al. (2004). This amount is approximately 100 times the amount considered to be a high dietary intake level of combined lutein and zeaxanthin relative to the average American diet (Seddon et al., 1994).

Another aim of this study was to examine the effectiveness of supplementation with equal amounts of lutein and zeaxanthin and to gain insight into the effects of 1:1 supplementation with these carotenoids and to identify whether there is an interaction.

Rhesus Macaque monkeys were selected as the animal model for this study because of their low serum xanthophylls concentrations relative to other species studied (Snodderly et al., 1990; Neuringer et al., 2004; Slifka et al., 1999; Crissey et al., 1999). Additionally, women have been shown to have lower MPOD than men (Hammond et al., 1996) and a higher risk of AMD (Klein et al., 1992; Pieramici et al., 1994), so the selection of female Rhesus monkeys posed the challenge of increasing plasma and ocular concentrations of lutein and zeaxanthin through dietary supplementation. As this study was nearing completion, another group of researchers reported on their studies in which Rhesus monkeys raised to adulthood on a xanthophyll-free diet were fed lutein and zeaxanthin as dietary supplements (Neuringer et al., 2004; Leung et al., 2004; Johnson et al., 2005; Leung et al., 2005). The mean serum concentrations reported for the control primates used in these studies was 74 ± 9 nmol/L for lutein and 81 ± 7 nmol/L for zeaxanthin, whereas the baseline levels in our study were considerably higher. The mean plasma lutein levels for the primates in our study were 240 ± 20 nmol/L for lutein and 120 ± 10 nmol/L for zeaxanthin. The diet selected for the primates in our study provided approximately 0.99–1.32 mg (1.74–2.32 μmol) of lutein, and 0.26–0.35 mg (0.46–0.62 μmol) of zeaxanthin per day, and the diet chosen in the primate studies conducted by Neuringer et al. provided nearly equal amounts of lutein (0.72–2.18 mg/day) and zeaxanthin (0.72–1.82 mg/day). The differences between the two diets cannot provide a reasonable explanation for the differences in serum or plasma concentrations of

carotenoids that were observed in the 2 studies. Both the mean age and mean body weight of the xanthophyll-free primate studies were nearly 3 times the mean values for the primates in our study which may have contributed to the disparities in serum/plasma carotenoid levels. At the outset of our study the animal weights ranged from 2.9 to 4.4 kg, and ages ranged from 1.9 to 3.9 years (Appendix J). The inter-individual variability with respect to weight and age was not significant, but there was considerable variability among the primates in their responses to the 3 treatments. The differences in baseline plasma levels suggest that younger Rhesus Macaque monkeys may absorb and metabolize lutein and zeaxanthin more efficiently than their older counterparts. However, because of these differences as well as differences in supplemental doses and duration, study design, and analytical procedures, the results of these studies are difficult to compare to those of our study. Where applicable in this thesis discussion, further comparisons of the results of these studies are made.

Changes in the Plasma Concentrations of Lutein, Zeaxanthin, and Their Metabolites in the Control, L-Treated, Z-Treated, and L/Z-Treated Primates

Plasma Concentrations of Lutein and Zeaxanthin

Mean plasma concentrations of both lutein and zeaxanthin in the L-treated group, the Z-treated group, and the L/Z-treated group increased during the initial 6 months of supplementation (Figure 23). It is important to note that the *all-trans*- and the *cis*-isomers of lutein (9-*cis*, 9'-*cis*, 13-*cis*, 13'-*cis*, 15-*cis*) and zeaxanthin (9-*cis*, 13-*cis*, 15-*cis*) were simultaneously separated by HPLC, but because the ratio of these isomers did not significantly change during the supplementation period and follow-up, the reported plasma concentrations of lutein and zeaxanthin are the combined totals for their

respective geometric isomers. See Table 16 for a summary of the mean plasma concentrations of lutein and zeaxanthin in the 3 treatment groups and the control group.

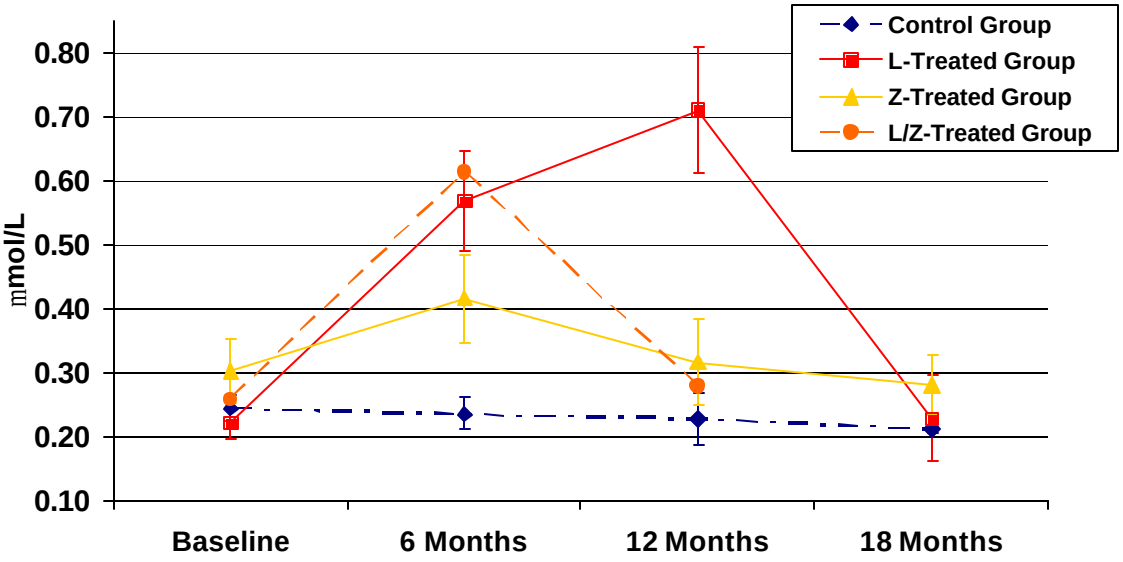


Figure 23. Changes in the mean plasma lutein concentrations (μmol/L) for the control, L-treated, Z-treated, and L/Z-treated primates at baseline and months 6, 12, and 18.

An increase in plasma lutein was expected in both the L-treated and L/Z-treated primates since the daily supplements contained 9.34 mg/kg and 0.5 mg/kg of lutein, respectively. This turned out to be the case, and in fact, it appeared that the bioavailability of lutein at month 6 was nearly equal for lutein at a daily dose of 9.34 mg/kg or 0.50 mg/kg. Interestingly, the mean plasma concentration of lutein in the Z-treated primates also increased although not significantly ($p = 0.07$) between baseline and month 6 of the supplementation period even though the zeaxanthin supplements did not contain any lutein. While it is possible that this initial increase in lutein during high-dose supplementation with zeaxanthin may have resulted from the *in vivo* transformation of zeaxanthin to lutein (Figure 2), it is not likely as zeaxanthin would have to undergo 2 conversions to reach this end point. This increase was more likely the result of the large

amount of inter-individual variability in plasma lutein levels of this group. It is also possible that the high concentration of circulating zeaxanthin may have enhanced the absorption of the dietary lutein that was provided in the stock diet.

Table 16. Mean concentrations of total lutein and total zeaxanthin in the plasma of control, L-treated, Z-treated, and L/Z-treated primates at baseline and months 6, 12, and 18.

Primate Group	Concentrations of Carotenoids (µmol/L) in Primate Plasma*							
	Lutein				Zeaxanthin			
	Baseline	6 Months	12 Months	18 Months	Baseline	6 Months	12 Months	18 Months
Control[a]								
Mean ± SEM	0.244 ± 0.021	0.235 ± 0.024	0.228 ± 0.041	0.212 ± 0.046	0.118 ± 0.009	0.069 ± 0.010	0.120 ± 0.027	0.120 ± 0.026
L-Treated[b]								
Mean ± SEM	0.223 ± 0.084	0.573 ± 0.062	0.709 ± 0.070	0.227 ± 0.052	0.111 ± 0.018	0.123 ± 0.003	0.147 ± 0.018	0.107 ± 0.027
Z-Treated[b]								
Mean ± SEM	0.303 ± 0.058	0.416 ± 0.071	0.316 ± 0.067	0.280 ± 0.046	0.230 ± 0.035	0.822 ± 0.075	0.918 ± 0.104	0.130 ± 0.067
L/Z-Treated[c]								
Mean ± SEM	0.258 ± 0.029	0.614 ± 0.061	0.278 ± 0.037	--[d]	0.111 ± 0.017	0.334 ± 0.040	0.136 ± 0.024	--[d]

* Lutein and zeaxanthin refer to the total combined amounts of *all-trans* plus the respective *cis*-isomers; [a] For the control group, n=3 at baseline, months 6 and 12; n=2 at month 18; [b] For the L-treated and Z-treated groups, n=5 at baseline, months 6 and 12; n=3 at month 18; [c] For the L/Z-treated primates, n=5 at baseline and months 6 and 12; [d] Data not available since primates were sacrificed at month 12.

The plasma lutein levels measured between months 6 and 12 of the supplementation period also yielded interesting results. While the mean plasma concentration of lutein increased significantly in the L-treated primates between months 6 and 12, the mean level for the L/Z-treated primates at month 12 (0.267 ± 0.037 µmol/L) was significantly lower than the 6 month value and was not significantly different from the mean baseline concentration (0.2581 ± 0.0294 µmol/L). Since daily supplementation with both lutein and zeaxanthin at a dose of 0.5 mg/kg of each continued during this time, it is possible that the organs and tissues absorbed more lutein after 6 months of supplementation. It appears

that chronic supplementation with the combination of lutein (0.5 mg/kg) and zeaxanthin (0.5 mg/kg) in primates does not result in persistent significant increases in plasma lutein levels. These results could also suggest an interaction between lutein and zeaxanthin when given at this dose that causes decreased absorption of lutein into the bloodstream after an initial peak is reached.

By comparing the L-treated group and the L/Z-treated group we can explore the possibility of an interaction caused by concurrent lutein and zeaxanthin supplementation. The L-treated primates, in addition to receiving a high dose of lutein (9.34 mg/kg) also received a lower daily dose of zeaxanthin (0.66 mg/kg) that was comparable to the dose given to the L/Z-treated group (0.5mg/kg). Because the lutein absorption into the plasma of the L-treated group did not appear to have been affected by the zeaxanthin supplementation between 6 and 12 months, it does not seem likely that zeaxanthin decreased the absorption of lutein into the bloodstream. Another interesting finding was that while the L/Z-treated group was given equal amounts of lutein and zeaxanthin (0.5 mg/kg each) plasma lutein levels were approximately twice as high as the zeaxanthin levels after 6 months of supplementation. While the higher mean plasma level of lutein at baseline compared to that of zeaxanthin can be explained by differences in the composition of the stock diet (L:Z $\cong$ 3.8), the 6 month levels cannot be as easily explained. It appears that lutein was better absorbed than zeaxanthin after 6 months of supplementation. This is a contrast to the higher mean levels of plasma zeaxanthin found at both 6 and 12 months in the primates supplemented daily with 10 mg/kg of zeaxanthin when compared to the levels of lutein in those supplemented daily with approximately the same amount of lutein (L-treated group).

Between baseline and 6 months only the Z-treated and the L/Z-treated primates had significant increases in their mean plasma zeaxanthin concentrations, even though the L-treated group received a dose of zeaxanthin slightly higher than the dose given to the L/Z-treated group. Table 16 and Figure 24 compare the mean zeaxanthin levels for the control and treatment groups (L, Z, and L/Z) during the supplementation period and after 6 months without supplementation.

While the L-treated primates were supplemented daily with approximately 0.66 mg/kg of zeaxanthin and the L/Z-treated primates received 0.5 mg/kg of zeaxanthin, the mean plasma concentration of zeaxanthin in the L/Z-treated group increased significantly ($p < 0.0001$) between baseline and month 6, but that of the L-treated group did not. Conversely, while the mean zeaxanthin level in the L-treated group was not significantly higher at month 6 compared to baseline, it was significantly higher between baseline and month 12 suggesting a gradual and consistent increase in plasma zeaxanthin concentration during the 12 months of supplementation. In the L/Z-treated primates, plasma zeaxanthin increased significantly (by 3-fold) between baseline and month 6, and then decreased to nearly the baseline concentration at month 12. Interestingly, the mean plasma concentrations of zeaxanthin at month 12 among the 2 groups were nearly the same. In the L/Z-treated primates a similar trend was observed in the changes in mean plasma lutein levels during 12 months of supplementation in that the concentration increased significantly between baseline and month 6 and declined thereafter to near the baseline level by month 12.

In comparing the mean plasma concentrations of lutein in the L-treated group (9.34 mg/kg L) and the L/Z-treated group (0.5 mg/kg L) at month 6 (Figure 23), it appears that supplementation with both a very high dose and a much lower dose of lutein

result in the same bioavailability of this carotenoid. Alternatively, examination of the mean zeaxanthin concentrations depicted in Figure 24 shows that this trend is not observed in of the primates supplemented with 0.5 mg/kg or 10 mg/kg of zeaxanthin. The mean plasma concentration of zeaxanthin in the L/Z-treated group was less than half that of the Z-treated group at month 6. Additionally, when lutein and zeaxanthin were given at the low dose of 0.5 mg/kg each, lutein was better absorbed than zeaxanthin. This suggests that lutein may interact with zeaxanthin to lower its bioavailability while the reverse does not seem to be the case.

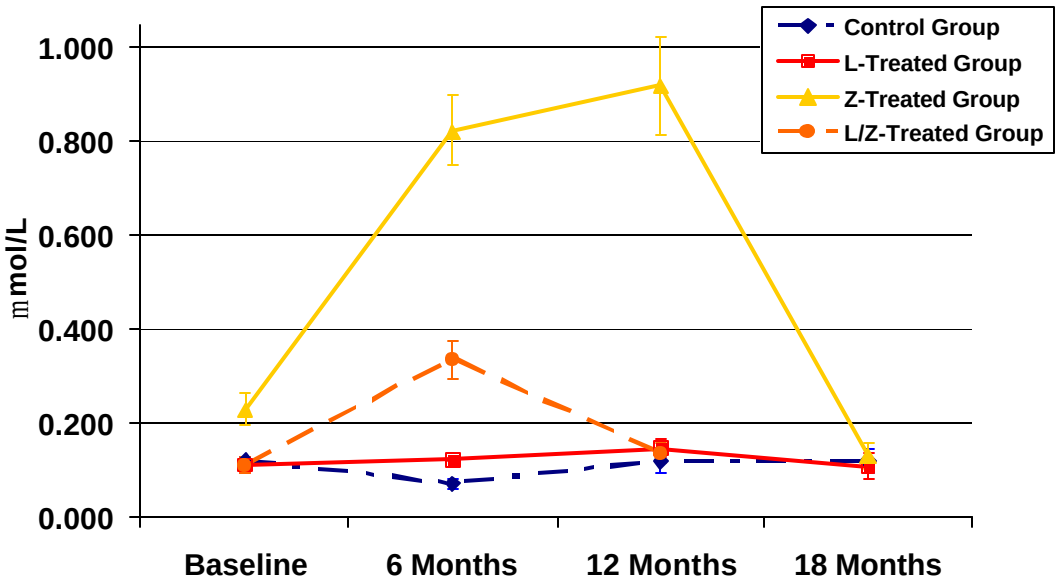


Figure 24. Changes in mean plasma zeaxanthin concentrations (µmol/L) in the control, L-treated, Z-treated, and L/Z-treated primates at baseline and months 6, 12, and 18.

In the Z-treated primates, the mean level of zeaxanthin in plasma increased by more than 3-fold between baseline and month 6, yet the increase between months 6 and 12 was not significant. This suggests that while mean plasma zeaxanthin levels increase rapidly with supplementation at this dose (10 mg/kg/day) and remain high, mean plasma zeaxanthin concentration plateaus at some point after 6 months of supplementation. By

month 18, the plasma level of zeaxanthin decreased to below the baseline level. However, the baseline concentration of zeaxanthin for this group was almost twice as high as the levels of the L-treated, L/Z-treated, and control groups and at 18 months, the mean level was nearly the same as those seen in the L-treated and Z-treated primates.

Comparing plasma concentrations of lutein or zeaxanthin in response to high-dose supplementation (~10 mg/kg/day) with these carotenoids provides an insight into the bioavailability of these carotenoids. Although the mean concentration for all of the primates in the study (n=18) at baseline was 0.259 µmol/L for lutein and 0.145 µmol/L for zeaxanthin, the mean concentration of lutein in the L-treated group (9.34 mg/kg of L) at month 6 (0.573 ± 0.062 µmol/L) was lower than that of zeaxanthin in the Z-treated group (10 mg/kg of Z) at month 6 (0.822 ± 0.075 µmol/L). Similarly, the mean level of lutein at month 12 in the L-treated group (0.709 ± 0.070 µmol/L) was also lower than that of zeaxanthin in the Z-treated group (0.918 ± 0.104 µmol/L). It is unlikely that the 0.66 mg/kg difference in supplemental dose could have caused this difference especially since the stock diet provided more lutein than zeaxanthin and could therefore balance this difference. These results suggest that zeaxanthin is more efficiently absorbed into the circulating blood than lutein and/or that lutein may be better absorbed into the tissues and organs than zeaxanthin, and is therefore sequestered by the tissues at a higher rate than zeaxanthin. It is particularly interesting to note that in the L/Z-treated primates that were fed equal doses of lutein and zeaxanthin (0.5 mg/kg of each), lutein appears to be more effectively absorbed into the bloodstream after 6 months of supplementation than zeaxanthin. This is clearly indicated by the mean concentration of lutein in plasma that was nearly twice that of zeaxanthin at month 6 (Figure 24). The doses that were given in

the present study were considerably higher than a dose that would be more appropriate for a clinical trial involving humans. Nonetheless, the possible interaction between supplemental lutein and zeaxanthin at various doses warrants further investigation.

Plasma Concentrations of Lutein and Zeaxanthin Metabolites

Based on evidence from earlier studies, it has been hypothesized that 3'-oxolutein is most likely formed via the allylic oxidation of dietary lutein (Khachik et al., 1995; Khachik et al, 1997a; Khachik et al., 1997c; Khachik et al., 2002). As shown in the proposed metabolic pathways in Figure 2, 3'-oxolutein can then be reduced to form 3'-epilutein or revert to lutein. It has also been proposed that dietary zeaxanthin can undergo stereospecific double-bond isomerization to form 3'-epilutein which can then be oxidized to form 3'-oxolutein. Given this overall scheme, it is possible that 3'-oxolutein and 3'-epilutein can be formed from lutein and/or zeaxanthin. It must be noted, however, that the validity of these proposed metabolic transformations can only be confirmed by supplementation studies with isotopically labeled lutein and zeaxanthin, which have not been conducted to date.

The mean plasma concentrations of 3'-oxolutein and 3'-epilutein in the control, L-treated, Z-treated, and L/Z-treated groups are compared in Figures 25 and 26, respectively. In the L-treated primates, the mean 3'-oxolutein concentration increased significantly between baseline and month 6, and continued to increase between months 6 and 12, although the latter change was not statistically significant. Meanwhile, the opposite effect was observed in the mean plasma concentration of 3'-epilutein. The increase in the mean level of 3'-epilutein in the L-treated group between baseline and

month 6 was not significant, while a statistically significant increase in the level of this metabolite occurred between months 6 and 12. These observations are consistent with the pathway proposed in Figure 2, and suggest that the oxidation of lutein to 3'-oxolutein within the first 6 months increases the level of this metabolite in the plasma to a point at which the stereo-controlled reduction of 3'-oxolutein to 3'-epilutein becomes significant.

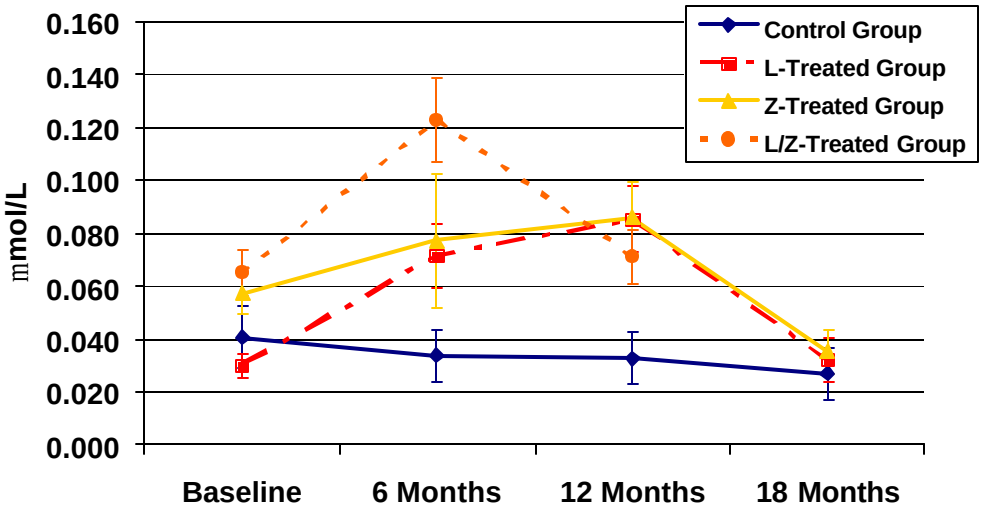


Figure 25. Changes in the mean plasma concentrations of 3'-oxolutein (µmol/L) in the control, L-treated, Z-treated, and L/Z-treated primates at baseline and at months 6, 12, and 18.

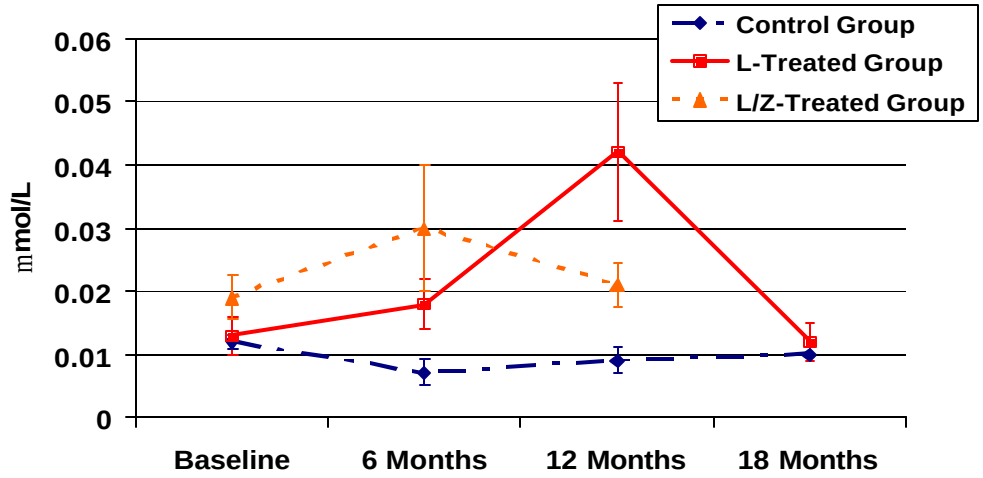


Figure 26. Changes in the mean plasma concentrations of 3'-epilutein (µmol/L) in the L-treated, L/Z-treated, and control primates. 3'-Epilutein was not consistently detected in the plasma of the Z-treated primates during the study and therefore this group was not included in this figure.

The mean plasma concentration of zeaxanthin in the Z-treated primates increased significantly within the first 6 months of supplementation ($p = 0.0002$), but appeared to plateau between months 6 and 12. Meanwhile, there was no significant change in the mean level of lutein in the plasma of the Z-treated primates between baseline and 12 months. The data pertaining to the concentration of 3'-epilutein in the Z-treated primates have been excluded from Figure 26 because only trace amounts of this metabolite were detected in some of the primates. Since daily supplementation with zeaxanthin at a dose of 10mg/kg does not appear to affect the mean plasma concentrations of 3'-epilutein, this may suggest that 3'-epilutein is predominantly formed from lutein but not zeaxanthin. Meanwhile, the mean plasma concentration of 3'-oxolutein in the Z-treated animals is significantly higher after 12 months of supplementation than that at baseline. However, the mean 3'-oxolutein level in plasma, much like that of zeaxanthin in the Z-treated group, seems to plateau after month 6. This pattern for plasma levels of 3'-oxolutein is also observed in the L-treated primates. Three primates from the L-treated and the Z-treated groups were observed for 6 months post-supplementation at which time mean plasma levels of these metabolites return to baseline levels.

The only significant change observed in the mean plasma metabolite concentrations in the L/Z-treated primates was that of 3'-oxolutein between baseline and month 6. Supplementation with lutein and zeaxanthin (each at 0.5 mg/kg) increases the mean concentrations of lutein and zeaxanthin after 6 months of supplementation, but then these levels decline to near their baseline values between months 6 and 12. This also appears to be the case with 3'-oxolutein levels in the plasma of the L/Z-treated group. Similarly, the mean plasma 3'-epilutein concentration in the L/Z-treated group increases between

baseline and month 6 and thereafter returns to its baseline value between months 6 and 12. The decrease in the mean concentrations of lutein, zeaxanthin, and their metabolites between months 6 and 12 in the L/Z-treated primates (0.5 mg/kg each of lutein and zeaxanthin) may suggest that during this period the supplemental carotenoids are better absorbed into the organs and tissues. The high concentrations of lutein, zeaxanthin, and 3'-oxolutein in the major organs and tissues of the supplemented animals at month 12 support this conclusion.

Concentrations of Lutein, Zeaxanthin, and Their Metabolites in the Ocular Tissues, Major Organs and Other Tissues of the Control and Supplemented Primates

Concentrations of Lutein, Zeaxanthin, and Their Metabolites in Ocular Tissues

Lutein and zeaxanthin were identified as the major carotenoids in the ocular tissues (retina, ciliary body, lens, iris) of the primates. The metabolites 3'-oxolutein and *meso*-zeaxanthin were detected in the retina while the former was also detected in the ciliary body. Due to the low concentration of carotenoids in the ciliary body, iris, and lens, no attempt was made to measure *meso*-zeaxanthin in these tissues. It has been proposed that 3'-oxolutein can be formed from lutein and/or zeaxanthin and that its presence in the ocular tissues may be the result of its transport from circulating blood or the *in vivo* enzymatic and light-induced metabolic transformation of lutein and/or zeaxanthin in the eye (Khachik et al., 1997a; Bernstein et al., 2001; Khachik et al., 1995). However, it has been demonstrated that *meso*-zeaxanthin, which is not of dietary origin is present in the retina yet is absent from both human plasma and liver (Khachik et al., 2002). Therefore, while the origin of 3'-oxolutein in the ocular tissues is unclear, *meso*-

zeaxanthin is most likely formed in the retina from lutein. Additional support for this metabolic transformation comes from the supplementation studies of xanthophyll-free primates by Johnson et al. (2005), who demonstrated that *meso*-zeaxanthin was absent in the retinas of xanthophyll-free and zeaxanthin-fed primates, but was present in the primates supplemented with lutein. These researchers showed that *meso*-zeaxanthin was present only in the macula (innermost 4-mm area of retina) of the primates that were supplemented with lutein (Johnson et al., 2005). One of the objectives of our study was to identify and quantify 3'-oxolutein and *meso*-zeaxanthin in the retina of the Rhesus monkeys that were supplemented with high doses of lutein and zeaxanthin. The central and peripheral regions of the retinas were not examined separately, because the low concentration of these metabolites in the various regions of the retina would not allow their unequivocal identification by HPLC-UV/Visible photodiode array detection. Consequently, the mean concentrations of carotenoids are expressed for the whole retina. Similarly, because the concentrations of carotenoids in the ciliary body, iris, and lens were very low, no attempt was made to quantify *meso*-zeaxanthin in these tissues.

After 12 months of supplementation, significant increases in the mean concentrations of lutein were found in the retina and iris of the L-treated primates in comparison to the control primates. The results depicted in Figure 27 clearly show the significant increases in the mean concentrations of lutein in the retina and iris of the primates that were supplemented daily with lutein (9.34 mg/kg L and 0.66 mg/kg Z) for 12 months in comparison with those of the control group. Six months after the end of supplementation with lutein (month 18), the mean level of lutein in the retina of the L-treated group was not significantly different than that of the control group ($p = 0.28$). The

mean concentrations of lutein in the ciliary body and lens after 12 months of daily supplementation with 9.34 mg/kg of lutein and 0.66 mg/kg of zeaxanthin did not change significantly compared to those of the control group although the mean level of lutein in lens increased in the animals that were sacrificed after 18 months. While the mean concentration of zeaxanthin in the retina of the L-treated primates at month 12 appeared to be much higher than that of the control group and the L-treated group at month 18, the differences were not statistically significant due to the large amount of inter-individual variability. The mean concentration of zeaxanthin in the ciliary body at month 12 was also higher than that of the control group, but this difference was not statistically significant. This is despite the fact that the mean plasma concentration of zeaxanthin is significantly increased after 12 months of supplementation with 0.66 mg/kg zeaxanthin in comparison to the mean baseline level. It should be noted, however, that the high inter-individual variability observed in the concentrations of this carotenoid was a limitation for establishing the true statistical significance.

There were significant increases in the mean concentrations of the metabolites 3'-oxolutein and *meso*-zeaxanthin in the retina of the L-treated (9.34 mg/kg L and 0.66 mg/kg Z) primates after 12 months of supplementation compared to that of their levels at month 18 (6 months post-supplementation) (Appendix K). There was no significant difference between the mean levels of *meso*-zeaxanthin in the primates supplemented with 9.34 mg/kg of lutein at month 12 and that of the control group. It should be noted that the mean concentration of *meso*-zeaxanthin in the control group was considerably higher than those of either the L-treated or the Z-treated (10 mg/kg Z) groups at month

18. Therefore the month 18 data for L-treated group may provide a better measure of the mean baseline level of *meso*-zeaxanthin.

A comparison of the mean plasma and retina concentrations of lutein in the L-treated (9.34 mg/kg L and 0.66 mg/kg Z) and the L/Z-treated (L and Z each at 0.5 mg/kg) compared to those of the control group are shown in Figure 27. It appears that the plasma and retina levels of this carotenoid are proportionally correlated at the 2 supplemental doses of 9.34 mg/kg and 0.5 mg/kg. A similar association between the mean lutein levels in the plasma and retina is observed in the control group that received no dietary supplement.

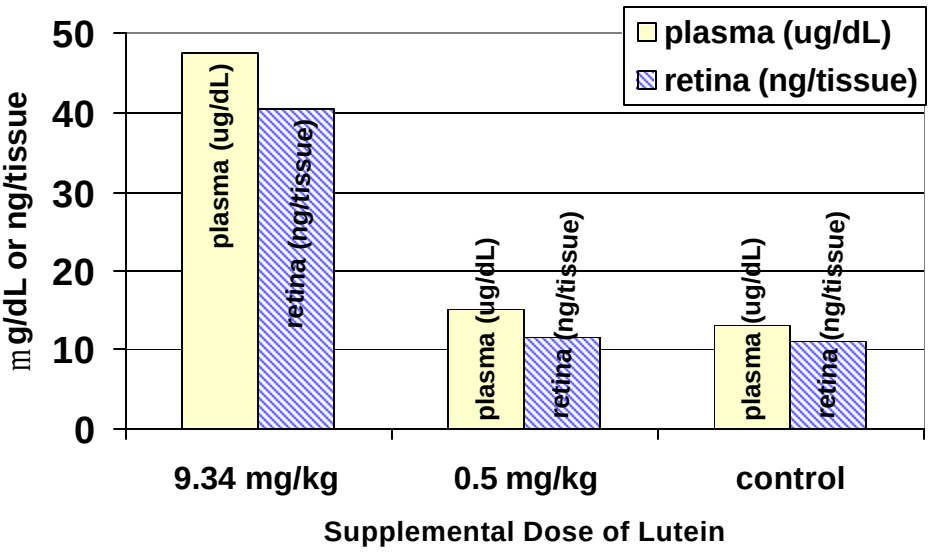


Figure 27. Mean plasma concentrations (µg/dL) and retina concentrations (ng/tissue) of lutein in the lutein-supplemented primates [L-treated (9.34 mg/kg), L/Z-treated (0.5 mg/kg)] at month 12 in comparison to the mean levels in the control primates.

The mean concentration of zeaxanthin in the retina of the Z-treated (10 mg/kg) monkeys increased by approximately 4-fold after 12 months compared to that of the animals in the control group. Supplementation with zeaxanthin for 12 months did not

increase the mean level of lutein in the retina of the Z-treated primates in comparison to the control group (11.05 ± 2.19 ng/tissue). However, the mean concentration of lutein in the retina of the Z-treated group at month 12 (19.2 ± 9.07 ng/tissue) was significantly higher than their mean level at month 18 (7.84 ± 1.03 ng/tissue). This may be attributed to the considerable amount of variability in the concentration of lutein in the retinas of this group in response to supplementation with zeaxanthin at a high dose. In addition, supplementation with zeaxanthin at the dose of 10 mg/kg significantly increases the mean concentration of this carotenoid in the ciliary body after 12 months in comparison to that of control animals.

Although the zeaxanthin supplements contained no lutein, the mean concentration of lutein in the retina of the Z-treated animals appeared to be considerably higher than that of the control animals. However, due to the large inter-individual variability in these levels, this difference was not significant. Comparisons of the mean concentrations of lutein, zeaxanthin, and their metabolites, *meso*-zeaxanthin and 3'-oxolutein, in the retinas among the control and the 3 treatment groups after 12 months of supplementation are depicted in Figure 28. See Appendix J for a summary of the mean concentrations of carotenoids and their metabolites in the ocular tissues for the study's four primate groups. It is interesting to note that the mean levels of lutein and zeaxanthin in the retina of the Z-treated group were nearly the same. This was a contrast to what was observed in the retinas of the L-treated group that had a mean lutein concentration that was more than 8-fold that of zeaxanthin. This indicates when Rhesus monkeys are given a dose of lutein or a comparable high dose of zeaxanthin, lutein accumulates in the retina more effectively than zeaxanthin.

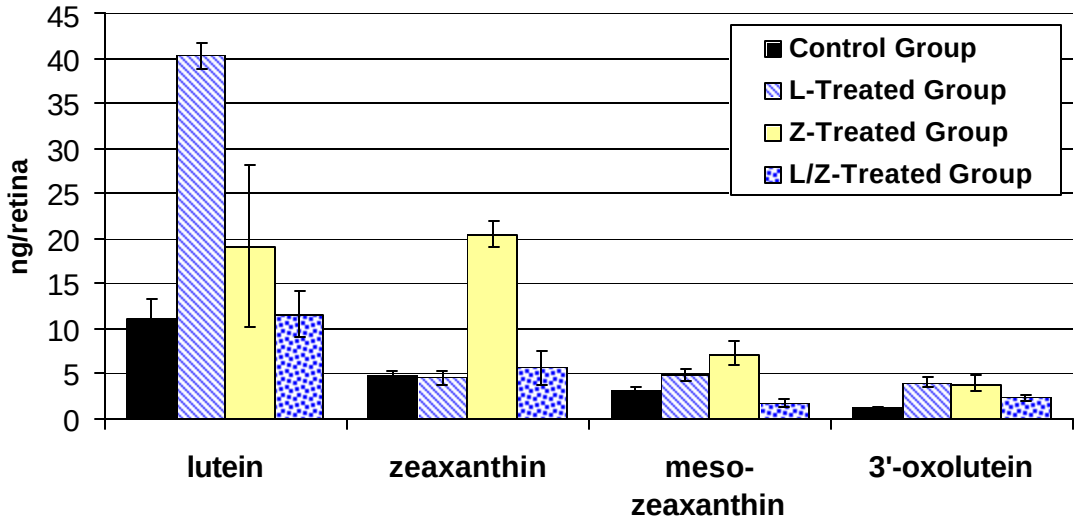


Figure 28. Mean concentrations (ng/tissue) of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein in the retina of the control, L-treated (9.34 mg/kg L and 0.66 mg/kg Z), Z-treated (10 mg/kg Z), and L/Z-treated (L & Z each at 0.5 mg/kg) primates at month 12.

The mean concentration of the metabolite 3'-oxolutein significantly increases in the retina after 12 months of supplementation with zeaxanthin at the dose of 10 mg/kg. In the ciliary body of these primates, however, no significant change in mean 3'-oxolutein concentration is observed. It is interesting that the mean level of *meso*-zeaxanthin was higher in the Z-treated (10 mg/kg) primates than in the L-treated (9.34 mg/kg L and 0.66 mg/kg Z) primates since it has been proposed that it is lutein and not zeaxanthin that is the major precursor for this metabolite. After 12 months of supplementation with 10 mg/kg of zeaxanthin, the mean concentration of *meso*-zeaxanthin in the retinas increases significantly. This contrasts the results of Johnson et al. who reported the absence of *meso*-zeaxanthin in the macula after supplementation of Rhesus monkeys with zeaxanthin. The supplemental dose given to those primates was less than one-fourth of that used in our study and the primates had been raised from birth on a xanthophyll-free

diet prior to supplementation. These differences make it increasingly difficult to compare results of the two studies.

In our study, we found that the mean level of *meso*-zeaxanthin in the retina of the L-treated primates is not significantly higher after 12 months when compared to the control group. Because of the large inter-individual variability, however, the month 18 values may be a better measure of the baseline level of this metabolite than the control value. Without metabolic studies that employ isotopically labeled lutein and zeaxanthin, it is not possible to determine the origin of *meso*-zeaxanthin with certainty. One possibility that cannot be ruled out is the formation of *meso*-zeaxanthin from 3'-oxolutein since the mean concentration of the latter metabolite was increased significantly after 12 months of daily supplementation with zeaxanthin (10 mg/kg) compared to the control primates. This metabolic conversion would require the stereospecific reduction of 3'-oxolutein to 3'-epilutein followed by double-bond migration. Because no measurable amount of 3'-epilutein was detected in the retina of the Z-treated primates, this transformation would most likely take place in a single step reaction. Alternatively, if *meso*-zeaxanthin is formed exclusively via double-bond isomerization of lutein, our results indicate that the high concentration of zeaxanthin in the retina of these primates may interact with lutein metabolism in this tissue. In both the iris and lens of the Z-treated primates, the mean lutein concentration was roughly 2 times as high at month 18 than at month 12, while zeaxanthin was detected at month 12 but not at month 18. These findings suggest the idea that zeaxanthin is cleared from both the iris and lens, while the concentrations of lutein in these tissues are actually higher at month 18 than at month 12.

The mean concentrations of the lutein and zeaxanthin in the retinas of the L/Z - treated group (L & Z each at 0.5 mg/kg) after 12 months of supplementation is not significantly different from that of the animals in the control group. This may be because the mean concentrations of these carotenoids in plasma of the monkeys peaked around month 6 and declined to their baseline values after this point. It appears that this decrease is not accompanied by an increase in the uptake of lutein and zeaxanthin by retina or ciliary body in Rhesus Macaque monkeys. Therefore, it is likely that lutein and zeaxanthin are incorporated into the retinas of the monkeys initially between baseline and month 6 of supplementation, and subsequently, as the mean plasma levels of these carotenoids decrease significantly between months 6 and 12, the retina levels follow the same pattern. The mean concentrations of *meso*-zeaxanthin and 3′-oxolutein in the retina of the L/Z treated animals after 12 months were not significantly different than those of the control group. This is not surprising since the levels of these metabolites in the retina of the monkeys appear to correlate with those of lutein and zeaxanthin. Figures 27 and 29 illustrate that plasma concentrations of lutein and zeaxanthin correlate directly with levels of these carotenoids in the retina. After 12 months of supplementation with lutein and zeaxanthin each at a dose of 0.5 mg/kg, only the concentrations of lutein and zeaxanthin appear to increase in the iris of the primates.

The high daily dose of supplemental lutein (9.34 mg/kg) led to a considerably higher mean concentration of this carotenoid (40.4 ± 1.41 ng/tissue) in the primate retinas compared to the mean zeaxanthin level (20.4 ± 3.88 ng/tissue) that resulted from daily supplementation with a comparable dose of zeaxanthin (10 mg/kg).

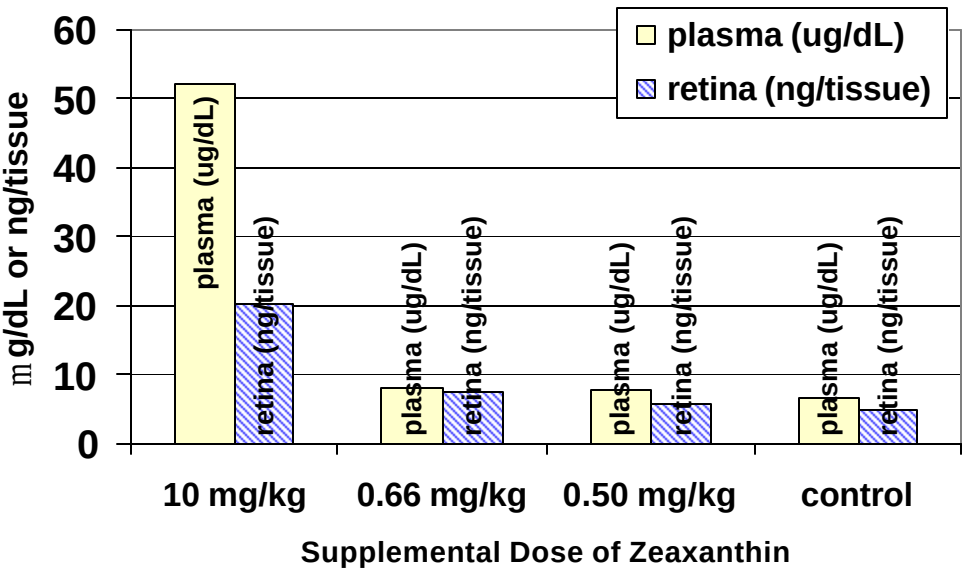


Figure 29. Mean concentration of zeaxanthin in plasma (µg/dL) and retina (ng/tissue) in the primates supplemented with zeaxanthin at month 12 in comparison to those of the control primates.

As shown in Figures 27 and 29, an interesting trend is clearly seen in the comparison of the changes in mean plasma carotenoid levels with the corresponding changes in the concentration of carotenoids in the retinas of the supplemented primates. The mean retina concentration of lutein in the L-treated, L/Z-treated, and control groups correlate well with the mean retina level of this carotenoid. However, in the Z-treated group that was fed a high-dose of zeaxanthin (10 mg/kg), the increase in mean concentration of this carotenoid in the retina was not as closely associated with the levels in plasma as were the levels of lutein in the L-treated primates. This may suggest that at a high supplemental dose of nearly 10 mg/kg, lutein may be better absorbed by the retina than zeaxanthin. This is despite the fact that the mean plasma concentrations of both carotenoids after 12 months of supplementation with lutein or zeaxanthin (~10 mg/kg) reach nearly the same concentration.

Concentrations of Lutein, Zeaxanthin and Their Metabolites in Major Organs and Other Tissues

Daily supplementation with a high dose of either lutein (9.34 mg/kg) or zeaxanthin (10 mg/kg) significantly increased the concentrations of lutein and zeaxanthin in most of the major organs and tissues of the primates after 12 months. For each group, the highest mean concentrations of these carotenoids were found in the liver. However, levels of lutein and zeaxanthin in the liver were considerably lower in the L/Z-treated group when compared to the L- and the Z-treated groups. This suggests that when plasma levels of lutein and/or zeaxanthin are chronically high the liver may act a storage repository for these carotenoids. Interestingly, the mean levels of lutein and zeaxanthin in the liver were significantly higher in the primates in the control group in comparison to those of the L/Z-treated group.

The mean concentrations of lutein and zeaxanthin in the lung were also much lower in the L/Z-treated group in comparison with those of the L-treated and the Z-treated groups. Additionally, the mean concentration of zeaxanthin in the lung tissues of the Z-treated group (1.65 µg/g) was nearly 7-fold that of lutein in the L-treated group (0.23 ± 0.02 µg/g). Similarly, in the colon the mean level of lutein in the L-treated group (0.23 ± 0.13 µg/g) was considerably lower than that of zeaxanthin in the Z-treated group (1.34 ± 0.78 µg/g). This suggests that while both carotenoids are effectively accumulated in the lung and colon tissue when administered at a high dose, zeaxanthin is absorbed more efficiently than lutein. The bioavailability of lutein and zeaxanthin in the other organs and tissues (liver, kidney, breast, ovary, spleen, cervix) appear to be about the same irrespective of the supplemental dose of lutein and zeaxanthin.

Lutein and zeaxanthin are also absorbed effectively into the major organs and tissues of the L/Z-treated monkeys (L & Z each at 0.5 mg/kg). The highest concentrations of these carotenoids were found in the liver, colon, breast, ovary, and spleen. In fact, at the dose of 0.5 mg/kg of lutein and zeaxanthin each, these carotenoids are equally as bioavailable in most organs and tissues of the primates as they are at the substantially higher dose of approximately 10 mg/kg of lutein or zeaxanthin fed separately (L-treated & Z-treated groups).

Overall, the the liver accumulates the highest levels of lutein and zeaxanthin among all 3 treatment groups as well as the control group. Both lutein and zeaxanthin are absorbed well into the organs and tissues of the primates, but the uptake of zeaxanthin into the lung and colon is considerably higher than that of lutein.

Limitations and Strengths of the Study

Small sample size was the major limitation of the study. Because there were only 3 control animals and 5 animals per treatment group it was difficult to detect small differences. The issue of small sample size was compounded by the high inter-individual variability that was observed in the concentrations of carotenoids in plasma and ocular tissues of the primates in response to supplementation. Larger treatment groups would have increased the statistical power of the tests (Analysis of Variance and Covariance, Least Significant Difference) that were applied to the data. The fact that the start dates for the three studies differed from one another by several months was another limitation. Furthermore, the control group was housed with the animals from the Lutein

Supplementation Study and followed the same timeline as this group. It is not known how this housing situation or the different start dates may have affected the results.

Another limitation lied in the variability in the weights of the retinas and other ocular tissues that were analyzed. It is not known whether weight is directly correlated to the total amount of carotenoids present in these tissues, and consequently, the extent to which this variability affected the data cannot be predicted.

In retrospect, a couple of aspects of the study design could have been modified to provide additional information. First, it would have been interesting to see the effects of treatment with a combined high dose of zeaxanthin (e.g., 10 mg/kg) and a low dose of lutein (e.g., 0.66 mg/kg); this would be opposite to the doses that were given to the L-treated group. Such a study would have been useful in further exploring the metabolic interaction between lutein and zeaxanthin in the plasma, retina, and various organs and tissues. Also, the study design could have been considerably improved by collecting additional blood samples between baseline, month 6, month 12 and month 18. This would in turn provide valuable information regarding the absorption of carotenoids and their metabolites into plasma and their rate of clearance from the circulating blood. Additionally, by increasing the number of animals in each group, it would have been possible to sacrifice several animals at month 6 and to correlate the concentrations of carotenoids and their metabolites in the ocular tissues to their corresponding levels in plasma. Unfortunately, the cost associated with procurement and long-term care of primates in this NEI-sponsored study did not allow for the inclusion of additional animals.

Conversely, this study has several noteworthy strengths. Rhesus Macaque monkeys have been shown to be a good animal model in that this species has similar carotenoid plasma and tissue deposition and metabolism to that of humans (Khachik et al., 1995). Therefore, the results obtained from the studies presented here can be extended to humans. Finally, the design of this study allowed us to measure the effects of long-term supplementation beyond the initial spikes in plasma carotenoid levels that have been shown to occur within a few weeks of supplementation (Khachik et al., 1995; Khachik et al., 1997c; Landrum et al., 1997b; Richer et al., 2004). By supplementing the animals with lutein and zeaxanthin at high doses for 12 months, we were able to determine the absorption of these carotenoids and their metabolites into the plasma, ocular tissues, and major organs and tissues, and establish their safety.

SUMMARY AND CONCLUSIONS

Daily supplementation of female Rhesus Macaque monkeys with lutein (9.34 mg/kg, 17.58 μmol/kg) for one year results in a 3.2-fold increase in the mean concentrations of this carotenoid in plasma and a 6.5-fold increase in that of the retina. Following treatment, these levels return to the baseline level after 6 months without further supplementation. This supplemental dose of lutein also results in 3.4- and 6-fold increases in the mean concentrations of *meso*-zeaxanthin and 3′-oxolutein in the retina of the monkeys, respectively.

Daily supplementation of Rhesus Macaque monkeys with zeaxanthin at nearly the same dose level (10 mg/kg) for one year increases the mean concentrations of zeaxanthin in the plasma and retina by 3.6-fold and 4.6-fold, respectively. These levels return to approximately the same levels measured at baseline by 6 months post-supplementation. Supplementation with a high-dose of zeaxanthin also results in significant increases in the mean levels of *meso*-zeaxanthin and 3'-oxolutein in the retina of the primates. These results suggest that although *meso*-zeaxanthin is most likely formed in the retina of the monkeys from dietary lutein, it may also be formed from 3'-oxolutein by the stereospecific reduction to 3′-epilutein followed by double bond isomerizaton.

Daily supplementation of primates with a combination of lutein and zeaxanthin (each at the dose of 0.5 mg/kg) for one year increases the mean plasma concentration of these carotenoids within the first 6 months, but thereafter, the levels of these carotenoids return to their baseline values. This is also reflected in the mean concentrations of lutein

and zeaxanthin in the retina of the L/Z-treated monkeys after 12 months that are not significantly different from the levels found in the control group.

Chronic supplementation of Rhesus Macaque monkeys with lutein, zeaxanthin or their 1:1 combination (0.5 mg/kg of each) increases the mean concentrations of these carotenoids in most of the major organs and major tissues, yet zeaxanthin is more efficiently absorbed than lutein in lung and colon when given at comparable doses. The highest accumulation of these carotenoids occurs in the liver regardless of the supplemental dose. The bioavailability of lutein and zeaxanthin in most of the major organs and tissues is nearly equal for this group (L & Z each at 0.5 mg/kg) in comparison with the groups that were supplemented with a high dose of these carotenoids. Retina histopathology revealed no ocular toxicity and measurement of biomarkers associated with kidney toxicity revealed no toxicity as well. This work was conducted by other co-investigators of the study presented in this thesis. Thus, supplementation of female Rhesus monkeys with lutein or zeaxanthin for one year at the dose of approximately 10 mg/kg (17.58 μmol/kg) does not cause ocular toxicity and has no effect on biomarkers associated with kidney toxicity.

This study provides a foundation for future clinical trials with a combination of lutein and zeaxanthin involving human subjects. For a 60 kg human, the chronic dose of lutein and zeaxanthin given to the L- and Z-treated primates is equivalent to 600 mg/day of lutein or zeaxanthin. Consequently, future long-term human supplementation studies with lutein and zeaxanthin that plan to investigate the efficacy of these carotenoids in the prevention of AMD at a much lower dose (e.g. 0.5 mg/kg or lower), should not present any problems associated with toxicity.

APPENDIX A. APPROVAL FROM INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

UNIVERSITY OF
MARYLAND
GRADUATE STUDIES AND RESEARCH
Institutional Animal Care & Use Committee

Michael C. Ma

Mm1@umail.umd.edu
phone: (301) 405-3941

July 15, 2004

Dr. Khachik
Chemistry Department
Bio-chem Building
CAMPUS

Dear Dr. Khachik:

This letter is to inform you that on **July 15, 2004**, the members of the Institutional Animal Care & Use committee (IACUC) approved your protocol renewal:

Chronic Ingestion of Lutein & Zeaxanthin in Primates

R-02-26A

Please note that this is the second (2) approved renewal for your protocol. An approved protocol is valid for one (1) year unless there is a change. Thus, this protocol is valid until **July 15, 2005.** Since you have renewed the protocol twice (2), you cannot renew the protocol after the latter date. If you wish to continue this protocol after that date, you **MUST** submit a new protocol.

Sincerely,

Michael C. Ma
Professor, Department of Entomology
Chair, IACUC

APPENDIX B. CAROTENOIDS IN THE OCULAR TISSUES OF L-TREATED PRIMATES AT MONTHS 12 AND 18

Concentrations (ng/tissue) of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein in ocular tissues of primates supplemented daily with lutein (10 mg/kg) after 12 months of treatment and after 18 months (12 months treatment plus 6 months follow-up)

Primate ID	**Concentrations (ng/tissue) of Carotenoids in Primates Ocular Tissues (Tissue Weight**, g)*								
	Retina				**Ciliary Body**			**Iris**	**Lens**
	lutein	**zeaxanthin**	***meso*-zea-xanthin**	**3'-oxo-lutein**	**lutein**	**zeaxanthin**	**3'-oxo-lutein**	**lutein**	**lutein**
Control									
RQ4079	14.52 (0.103)	5.35 (0.103)	3.25 (0.103)	1.33 (0.103)	4.25 (0.0707)	2.00 (0.0707)	0.651 (0.0707)	0.391 (0.0160)	0.771 (0.136)
RQ4107	11.63 (0.137)	4.31 (0.137)	3.69 (0.137)	1.14 (0.137)	4.13 (0.0598)	2.11 (0.0598)	0.668 (0.0598)	0.401 (0.0111)	0.437 (0.218)
RQ4142	6.99 (0.111)	3.54 (0.111)	2.66 (0.111)	N.D.	3.70 (0.0419)	0.907 (0.0419)	0.472 (0.0419)	N.D.	0.576 (0.0646)
Mean ± SEM	**11.05±2.19 (0.117)**	**4.78±0.62 (0.117)**	**3.20±0.30 (0.117)**	**1.24±0.10 (0.117)**	**4.03±0.17 (0.0576)**	**1.67±0.38 (0.0576)**	**0.597±0.06 (0.0576)**	**0.40±0.01 (0.0115)**	**0.60±0.10 (0.140)**
L Treatment 12 months									
RQ4087	41.76 (0.110)	8.26 (0.110)	5.57 (0.110)	3.42 (0.110)	7.34 (0.063)	1.26 (0.063)	1.03 (0.063)	2.58 (0.027)	0.68 (0.147)
RQ4122	38.94 (0.129)	6.85 (0.129)	4.17 (0.129)	4.52 (0.129)	6.90 (0.029)	2.42 (0.029)	1.15 (0.029)	0.77 (0.020)	0.83 (0.182)
Mean ± SEM	**40.35±1.30 (0.120)**	**7.56±0.71 (0.120)**	**4.87±0.70 (0.120)**	**3.97±0.55 (0.120)**	**7.12±0.22 (0.046)**	**1.84±0.58 (0.046)**	**1.09±0.06 (0.046)**	**1.68±0.91 (0.024)**	**0.76±0.08 (0.165)**

* Weight of tissues are shown in parentheses (grams); N.D., not detected.

APPENDIX B. (continued)

Primate ID	Concentrations (ng/tissue) of Carotenoids in Primates Ocular Tissues (Tissue Weight, g)*								
	Retina				Ciliary Body			Iris	Lens
	lutein	zeaxanthin	*meso*-zea-xanthin	3'-oxo-lutein	lutein	zeaxanthin	3'-oxo-lutein	lutein	lutein
L Treatment 18 months									
RQ4078	7.49 (0.0609)	2.78 (0.0609)	2.32 (0.0609)	0.80 (0.0609)	2.81 (0.0673)	1.08 (0.0673)	N.D	0.78 (0.0020)	1.42 (0.0019)
RQ4175	5.08 (0.0405)	0.939 (0.0405)	0.821 (0.0405)	0.54 (0.0405)	6.00 (0.0545)	2.12 (0.0545)	N.D.	0.40 (0.0085)	0.75 (0.1041)
RQ4189	6.03 (0.0312)	3.40 (0.0312)	1.11 (0.0312)	0.65 (0.0312)	2.12 (0.0350)	0.98 (0.0350)	N.D.	N.D.	1.90 (0.0839)
Mean ± SEM	**6.20±0.70 (0.0442)**	**2.34±0.78 (0.0442)**	**1.45±0.44 (0.0442)**	**0.66±0.08 (0.0442)**	**3.64±1.20 (0.0523)**	**1.39±.0.36 (0.0523)**	**N.D.**	**0.59±0.19 (0.0053)**	**1.36±0.33 (0.0423)**

* Weight of tissues are shown in parentheses (grams); N.D., not detected.

APPENDIX C. CAROTENOIDS IN THE MAJOR ORGANS AND TISSUES OF THE L-TREATED PRIMATES

Concentrations (µg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in organs and tissues of one control and 2 L-treated primates (10 mg/kg/day of lutein) for 12 months.

Tissue	**Primate ID**		**Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues***			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Liver						
	Control	RQ4079	1.653	0.7751	N.D.	11.331
	Treatment	RQ4122	3.644	1.211	N.D.	11.999
		RQ4087	2.508	0.625	N.D.	28.806
		Mean ± SEM	**3.076 ± 0.57**	**0.918 ± 0.29**	**N.D.**	**20.402±8.40**
Lung						
	Control	RQ4079	0.100	0.051	0.006	0.164
	Treatment	RQ4122	0.250	0.063	0.027	0.061
		RQ4087	0.207	0.041	0.022	0.148
		Mean±SEM	**0.228 ± 0.02**	**0.052 ± 0.01**	**0.024 ± 0.002**	**0.104±0.044**
Colon						
	Control	RQ4079	0.090	0.060	0.036	0.234
	Treatment	RQ4122	0.360	0.140	0.049	0.114
		RQ4087	0.102	0.027	0.127	0.173
		Mean±SEM	**0.231 ± 0.13**	**0.084 ± 0.05**	**0.088 ± 0.04**	**0.144±0.030**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; [a] Concentrations of carotenoids and retinol for ovaries are expressed in nmol/0.1 g of ovaries rather than nmol/g of tissue; [b] Right and left tissues have been designated with (r), (l), respectively; N.D., Not detected.

APPENDIX C. (continued)

Tissue	Primate ID		Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues*			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Kidney						
	Control	RQ4079	0.049	0.027	0.005	1.442
	Treatment	RQ4122	0.271	0.105	0.042	1.228
		RQ4087	0.139	0.044	0.029	1.268
		Mean ± SEM	**0.205 ± 0.07**	**0.074 ± 0.03**	**0.035 ± 0.007**	**1.249±0.026**
Breast[a]						
	Control	RQ4079(r)	0.020	0.012	0.006	0.023
		RQ4079 (l)	0.054	0.028	0.012	0.060
		Mean ± SEM	**0.037 ± 0.017**	**0.020 ± 0.008**	**0.009 ± 0.003**	**0.032±0.021**
	Treatment	RQ4122 (r)	0.549	0.134	0.101	0.059
		RQ4122 (l)	0.701	0.177	0.143	0.063
		RQ4087(r)	0.085	0.019	0.015	0.032
		RQ4087 (l)	0.113	0.064	0.018	0.061
		Mean ± SEM	**0.362 ± 0.26**	**0.098 ± 0.06**	**0.069 ± 0.05**	**0.054±0.007**
Ovaries[b]						
	Control	RQ4079	0.026	0.017	0.003	0.033
	Treatment	RQ4122	0.046	0.019	0.004	0.054
		RQ4087	0.096	0.019	0.021	0.061
		Mean ± SEM	**0.071 ± 0.03**	**0.019 ± 0.003**	**0.013 ± 0.008**	**0.058±0.004**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; [a] Concentrations of carotenoids and retinol for ovaries are expressed in nmol/0.1 g of ovaries rather than nmol/g of tissue; [b] Right and left tissues have been designated with (r), (l), respectively; N.D., Not detected.

APPENDIX C. (continued)

Tissue	Primate ID		Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues*			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Spleen						
	Control	RQ4079	0.322	0.138	0.029	0.134
	Treatment	RQ4122	0.679	0.163	0.073	0.139
		RQ4087	0.561	0.123	0.052	0.110
		Mean ± SEM	**0.620 ± 0.06**	**0.143 ± 0.02**	**0.062 ± 0.01**	**0.125 ± 0.015**
Cervix	Control	RQ4079	0.017	0.013	0.002	0.028
	Treatment	RQ4122	0.037	0.014	0.011	0.020
		RQ4087	0.065	0.034	N.D.	0.094
		Mean±SEM	**0.051 ± 0.01**	**0.019 ± 0.01**	**0.011 ± 0.004**	**0.057 ± 0.037**
Adipose	Control	RQ4079	0.163	0.095	0.072	0.147
	Treatment	RQ4122	1.539	0.346	0.407	0.133
		RQ4087	0.050	0.027	N.D.	N.D.
		Mean±SEM	**0.794 ± 0.82**	**0.187 ± 0.16**	**0.407**	**0.133**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; [a] Concentrations of carotenoids and retinol for ovaries are expressed in nmol/0.1 g of ovaries rather than nmol/g of tissue; [b] Right and left tissues have been designated with (r), (l), respectively; N.D., Not detected.

APPENDIX D. CAROTENOIDS IN THE MAJOR ORGANS AND TISSUES OF L-TREATED PRIMATES AT MONTH 18

Concentrations (μg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in organs and tissues 6 months post-supplementation (month 18) of 2 control primates and 3 primates supplemented daily with lutein (10 mg/kg) for 12 months.

Tissue	Primate ID		Concentrations (μg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues*			
			Lutein	**Zeaxanthin**	**3'-Oxolutein**	**Retinol**
Liver	**Control**	RQ4107	1.374	0.873	N.D.	28.309
		RQ4142	0.499	0.282	N.D.	41.894
		Mean ±SEM	**0.937 ± 0.44**	**0.577 ± 0.30**	**N.D.**	**35.101 ± 6.79**
	Treatment	RQ4078	1.261	0.709	0.028	25.781
		RQ4175	3.963	1.093	0.042	20.680
		RQ4189	0.795	0.433	N.D.	29.925
		Mean±SEM	**2.006 ± 0.99**	**0.745 ± 0.19**	**0.035 ± 0.007**	**25.461 ± 2.67**
Lung**	**Control**	RQ4107	0.101	0.055	0.011	0.093
		RQ4142	0.104	0.064	0.009	0.095
		Mean±SEM	**0.102 ± 0.002**	**0.059 ± 0.005**	**0.010 ± 0.001**	**0.094 ± 0.001**
	Treatment	RQ4078	0.084	0.040	0.009	0.181
		RQ4175	0.244	0.106	0.034	0.193
		RQ4189	0.080	0.039	0.011	0.159
		Mean±SEM	**0.136 ± 0.12**	**0.061 ± 0.022**	**0.018 ± 0.008**	**0.177 ± 0.010**

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; ** Trace amounts of 3'-epilutein detected; N.D., Not detected.

APPENDIX D. (continued)

Tissue	Primate ID		Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein,and Retinol in Primate Tissues*			
			Lutein	**Zeaxanthin**	**3'-Oxolutein**	**Retinol**
Colon	**Control**	RQ4107**	0.223	0.137	0.071	0.354
		RQ4142**	0.289	0.152	0.039	0.198
		Mean±SEM	**0.256 ± 0.033**	**0.144 ± 0.008**	**0.055 ± 0.016**	**0.276 ± 0.078**
	Treatment	RQ4078**	0.195	0.081	0.040	0.132
		RQ4175**	0.314	0.128	0.057	0.365
		RQ4189**	0.090	0.033	0.017	0.154
		Mean±SEM	**0.199 ± 0.065**	**0.081 ± 0.027**	**0.038 ± 0.012**	**0.217 ± 0.074**
Breast	**Control**	RQ4107**	0.624	0.417	0.158	0.285
		RQ4142**	0.290	0.160	0.092	0.135
		Mean±SEM	**0.457 ± 0.17**	**0.289 ± 0.24**	**0.125 ± 0.033**	**0.210 ± 0.075**
	Treatment	RQ4078**	0.234	0.083	0.081	0.056
		RQ4175**	0.332	0.104	0.121	0.048
		RQ4189**	0.476	0.178	0.209	0.199
		Mean±SEM	**0.347 ± 0.053**	**0.121 ± 0.029**	**0.137 ± 0.038**	**0.084 ± 0.051**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; ** Trace amounts of 3'-epilutein detected; N.D., Not detected.

APPENDIX E. CAROTENOIDS IN THE OCULAR TISSUES OF Z-TREATED PRIMATES AT MONTHS 12 AND 18

Concentrations (ng/tissue) of lutein, zeaxanthin, *meso*-zeaxanthin and 3'-oxolutein in ocular tissues of 5 primates supplemented daily with zeaxanthin (10 mg/kg) at month 12 (n=2) and at 6 months post-supplementation (n=3) compared to 3 control primates.

Primate ID	**Concentrations (ng/tissue) of Carotenoids in Primates Ocular Tissues (Tissue Weight, g)[1]**								
	Retina				**Ciliary Body**			**Iris**	**Lens**
	lutein	**zea-xanthin**	***meso*-zea-xanthin**	**3'-oxo-lutein**	**lutein**	**zea-xanthin**	**3'-oxo-lutein**	**lutein/ zeaxanthin**	**lutein/ zeaxanthin**
Control Group									
RQ4079	14.52 (0.103)	5.35 (0.103)	3.25 (0.103)	1.33 (0.103)	4.25 (0.071)	2.00 (0.071)	0.65 (0.071)	0.39/00 (0.016)	0.77/00 (0.136)
RQ4107	11.63 (0.137)	5.45 (0.137)	3.64 (0.137)	1.14 (0.137)	4.13 (0.060)	2.11 (0.060)	0.668 (0.060)	0.40/00 (0.0111)	0.44/00 (0.218)
RQ4142	6.99 (0.111)	3.54 (0.111)	2.66 (0.111)	N.D.	3.70 (0.042)	0.907 (0.042)	0.472 (0.042)	N.D.	0.58/.532 (0.065)
Mean ± SEM	**11.05± 2.19 (0.117)**	**4.78± 0.62 (0.117)**	**3.20± 0.30 (0.117)**	**0.82± 0.10 (0.117)**	**4.03± 0.17 (0.058)**	**1.67± 0.38 (0.058)**	**0.60± 0.06 (0.058)**	**0.26±.01/ N.D. (0.012)**	**0.60±.10/ 0.18±.05 (0.140)**
Treatment (Z) Group (12 months)									
RQ4146	28.19 (0.184)	24.30 (0.184)	8.56 (0.184)	4.69 (0.184)	7.51 (0.049)	17.12 (0.049)	1.83 (0.049)	0.31/0.39 (0.034)	0.34/0.32 (0.163)
RQ4173	10.10 (0.126)	16.56 (0.126)	5.83 (0.126)	3.05 (0.126)	2.42 (0.057)	8.58 (0.057)	0.84 (0.057)	0.21/0.20 (0.021)	0.33/0.41 (0.163)
Mean ± SEM	**19.15± 9.07 (0.105)**	**20.43± 3.88 (0.105)**	**7.20± 1.37 (0.105)**	**3.87± 0.10 (0.105)**	**4.97± 2.55 (0.053)**	**12.85± 4.28 (0.053)**	**1.34± 0.50 (0.053)**	**0.26±.05/ .30±.01 (0.028)**	**0.34±.01/ .37±.05 (0.163)**

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; N.D., Not detected.

APPENDIX E. (continued)

Primate ID	Concentrations (ng/tissue) of Lutein, Zeaxanthin, *meso*-Zeaxanthin, and 3'-Oxolutein in Primates Ocular Tissues (Tissue Weight, g)[1]								
	Retina				Ciliary Body			Iris	Lens
	lutein	zeaxanthin	*meso*-zea-xanthin	3'-oxo-lutein	lutein	zea-xanthin	3'-oxo-lutein	lutein/ zeaxanthin	lutein/ zeaxanthin
Treatment (Z) Group (18 months)									
RQ4092 **(right)**	13.31 (0.056)	4.50 (0.056)	2.32 (0.056)	1.56 (0.056)	8.12 (0.075)	4.91 (0.075)	1.04 (0.075)	0.88/00 (0.012)	0.87/00 (0.118)
(left)	5.81 (0.061)	3.48 (0.061)	1.43 (0.061)	0.629 (0.061)	10.32 (0.076)	4.37 (0.076)	1.34 (0.076)	0.40/00 (0.009)	0.48/00 (0.146)
RQ4094 **(right)**	11.13 (0.044)	3.70 (0.044)	0.92 (0.044)	N.D.	5.85 (0.074)	2.94 (0.074)	0.87 (0.074)	N.A.	0.40/00 (0.083)
(left)	0.852 (0.011)	N.D.	N.D.	N.D.	7.06 (0.102)	4.00 (0.102)	1.14 (0.102)	0.70/00 (0.009)	0.47/00 (0.169)
RQ4120 **(right)**	10.92 (0.054)	6.51 (0.054)	1.98 (0.054)	0.57 (0.054)	7.13 (0.116)	2.91 (0.116)	0.78 (0.116)	0.90/00 (0.008)	1.02/ 00 (0.163)
(left)	5.03 (0.020)	5.14 (0.020)	2.23 (0.020)	0.57 (0.020)	4.57 (0.083)	2.06 (0.083)	0.55 (0.083)	0.35/00 (0.013)	0.54/00 (0.155)
Mean ± SEM	**7.842± 1.03 (0.041)**	**3.93± 0.66 (0.041)**	**1.78± 0.26 (0.041)**	**0.58± 0.27 (0.041)**	**7.18± 1.04 (0.095)**	**3.53± 0.62 (0.095)**	**0.95± 0.19 (0.095)**	**0.65±.01/ N.D (0.013)**	**0.63± .01/ N.D. (0.169)**

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding *all-trans* isomers; N.D., Not detected.

APPENDIX F. CAROTENOIDS IN THE MAJOR ORGANS AND TISSUES OF Z-TREATED PRIMATES AT MONTH 12

Concentrations (µg/g tissues) of lutein, zeaxanthin, 3'-oxolutein, and retinol in tissues of one control and 2 primates supplemented daily with zeaxanthin (10 mg/kg) at month 12.

Tissue	Primate ID		Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues*			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Liver	Control	RQ4079	1.653	0.775	N.D.	11.331
	Treatment	RQ4146	2.723	4.718	0.191	11.739
		RQ4173	0.74	3.063	0.103	10.492
		Mean ± SEM	**1.732 ± 0.991**	**3.890 ± 0.828**	**0.147 ± 0.044**	**11.116 ± 0.623**
Lung	Control	RQ4079	0.100	0.051	0.006	0.164
	Treatment	RQ4146	0.052	0.115	0.008	0.020
		RQ4173	0.052	0.216	0.018	0.144
		Mean ± SEM	**0.052 ± 0.00**	**0.166 ± 0.051**	**0.013 ± 0.009**	**0.082± 0.062**
Colon	Control	RQ4079	0.090	0.060	0.036	0.234
	Treatment	RQ4146	0.503	2.114	0.023	0.034
		RQ4173	0.073	0.562	0.023	0.040
		Mean ± SEM	**0.288 ± 0.215**	**1.085 ± 1.468**	**0.023 ± 0.00**	**0.036 ± 0.003**
Breast [a]	Control	RQ4079(r)	0.020	0.012	0.006	0.023
		RQ4079(l)	0.054	0.028	0.012	0.060
		Mean ± SEM	**0.037 ± 0.017**	**0.020 ± 0.008**	**0.009 ± 0.003**	**0.042 ± 0.019**
	Treatment	RQ4146 (r)	0.086	0.328	0.041	0.045
		RQ4146 (l)	0.047	0.227	0.017	0.031
		RQ4173 (r)	0.149	0.575	0.077	0.054
		RQ4173 (l)	0.042	0.329	0.042	0.038
		Mean ± SEM	**0.081± 0.025**	**0.36 5± 0.074**	**0.044 ± 0.012**	**0.042 ± 0.0045**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; [a] Right and left tissues have been designated with (r), (l), respectively; [b]Concentrations of carotenoids and retinol for ovaries are expressed in µg/0.1 g of ovaries rather than µg/g of tissue; N.D., Not detected.

APPENDIX F. (continued)

Tissue	Primate ID		Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primates' Tissues*			
			lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Kidney	Control	RQ4079	0.049	0.027	0.005	1.442
	Treatment	RQ4146	0.084	0.161	0.016	1.093
		RQ4173	0.025	0.089	0.08	1.204
		Mean ± SEM	**0.055 ± 0.029**	**0.125 ± 0.036**	**0.012 ± 0.004**	**1.149 ± 0.056**
Ovaries[b]	Control	RQ4079	0.026	0.017	0.003	0.033
						0.013
	Treatment	RQ4146	0.014	0.028	0.001	0.020
		RQ4173	0.012	0.054	0.003	
		Mean ± SEM	**0.013 ± 0.001**	**0.041 ± 0.014**	**0.002 ± 0.001**	**0.016 ± 0.004**
Spleen	Control	RQ4079	0.322	0.138	0.029	0.134
	Treatment	RQ4146	0.277	0.723	0.026	0.041
		RQ4173	0.060	0.227	0.017	0.049
		Mean ± SEM	**0.169 ± 0.109**	**0.475 ± 0.351**	**0.022 ± 0.004**	**0.045 ± 0.004**
Cervix	Control	RQ4079	0.017	0.013	0.002	0.028
	Treatment	RQ4146	0.071	0.114	0.013	0.034
		RQ4173	0.015	0.050	0.005	0.039
		Mean ± SEM	**0.043 ± 0.028**	**0.082 ± 0.032**	**0.009 ± 0.004**	**0.037 ± 0.003**
Adipose	Control	RQ4079	0.163	0.095	0.072	0.147
	Treatment	RQ4146	0.066	0.174	0.026	0.020
		RQ4173	0.014	0.077	0.006	0.014
		Mean ± SEM	**0.040 ± 0.026**	**0.125 ± 0.048**	**0.016 ± 0.010**	**0.017 ± 0.003**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; [a] Right and left tissues have been designated with (r), (l), respectively; [b]Concentrations of carotenoids and retinol for ovaries are expressed in µg/0.1 g of ovaries rather than µg/g of tissue; N.D. Not detected.

APPENDIX G. CAROTENOIDS IN THE MAJOR ORGANS AND TISSUES OF Z-TREATED PRIMATES AT MONTH 18

Concentrations (µg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in organs and tissues 6 months post-supplementation (month 18) of 3 primates supplemented daily with zeaxanthin (10 mg/kg) for 12 months and 2 control primates.

Tissue	Primate ID	Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues			
		lutein	zeaxanthin	3'-oxolutein	retinol
Liver	**Control**				
	RQ4107	1.374.34	0.872.85	N.D.	28.309.3
	RQ4142	0.498.71	0.281.53	N.D.	41.893.9
	Mean ± SEM	**0.936.53 ± 0.536.53**	**0.577.19 ± 0.295.7**		**35.101.2 ± 6.792.1**
	Treatment				
	RQ4092**	1.572.42	0.756.01	N.D.	108.348
	RQ4094**	2.355.63	0.999.87	N.D.	209.662
	RQ4120	0.901.87	0.483.07	N.D.	124.720
	Mean ± SEM	**1.609.97 ± 0.132.8**	**0.746.32 ± 0.149.3**		**147.577 ± 31.400**
Lung**	**Control**				
	RQ4107	0.101	0.055	0.011	0.093
	RQ4142	0.104	0.064	0.009	0.095
	Mean±SEM	**0.102 ± 0.065**	**0.059 ± 0.005**	**0.010 ± 0.001**	**0.094 ± 0.001**
	Treatment				
	RQ4092	0.172	0.088	0.016	0.142
	RQ4094	0.142	0.072	0.015	0.173
	RQ4120	0.140	0.074	0.010	0.258
	Mean±SEM	**0.152 ± 0.010**	**0.078 ± 0.005**	**0.013 ± 0.002**	**0.191 ± 0.035**

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; ** Trace amounts of 3'-epilutein detected; N.D., Not detected.

APPENDIX G. (continued)

Tissue	Primate ID	Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues			
		lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Colon**	**Control**				
	RQ4107	0.223	0.137	0.071	0.354
	RQ4142	0.289	0.152	0.039	0.198
	Mean±SEM	**0.256 ± 0.033**	**0.144 ± 0.121**	**0.055 ± 0.016**	**0.276 ± 0.078**
	Treatment				
	RQ4092	0.349	0.193	0.039	0.168
	RQ4094	0.123	0.037	0.018	0.207
	RQ4120	0.327	0.177	0.021	0.337
	Mean±SEM	**0.266 ± 0.072**	**0.135 ± 0.050**	**0.026 ± 0.007**	**0.237 ± 0.051**
Breast**	**Control**				
	RQ4107	0.624	0.417	0.158	0.285
	RQ4142	0.290	0.160	0.092	0.135
	Mean±SEM	**0.457 ± 0.0167**	**0.289 ± 0.129**	**0.125 ± 0.033**	**0.210 ± 0.075**
	Treatment				
	RQ4092	0.117	0.074	0.030	0.040
	RQ4094	0.163	0.130	0.057	0.033
	RQ4120	0.399	0.329	0.105	0.284
	Mean±SEM	**0.227 ± 0.088**	**0.178 ± 0.077**	**0.064 ± 0.022**	**0.119 ± 0.083**

* Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; ** Trace amounts of 3'-epilutein detected; N.D.:, Not detected.

APPENDIX H. CAROTENOIDS IN THE OCULAR TISSUES OF L/Z-TREATED PRIMATES AT MONTH 12

Concentrations (ng/tissue) of lutein, zeaxanthin, *meso*-zeaxanthin, and 3'-oxolutein in ocular tissues of 3 control and 5 L/Z-treated primates (each at 0.5 mg/kg) at month 12.

Primate ID	**Concentrations (ng/tissue) of Lutein, Zeaxanthin, *meso*-Zeaxanthin, and 3'-Oxolutein in Primates' Ocular Tissues (Tissue Weight, g)***										
	Retina				**Ciliary Body**			**Iris**		**Lens**	
	lutein	**zea-xanthin**	***meso*-zea-xanthin**	**3'-oxo-lutein**	**lutein**	**zea-xanthin**	**3'-oxo-lutein**	**lutein**	**zea-xanthin**	**lutein**	**zea-xanthin**
Control											
RQ4079	14.52 (0.103)	5.35 (0.103)	3.25 (0.103)	1.33 (0.103)	4.25 (0.071)	2.00 (0.071)	0.65 (0.071)	0.39 (0.016)	N.D.	0.77 (0.136)	N.D.
RQ4142	6.99 (0.113)	3.54 (0.113)	2.66 (0.113)	N.D.	3.697 (0.042)	0.91 (0.042)	0.47 (0.042)	N.D.	N.D.	0.58 (0.065)	0.53 (0.065)
RQ4107	11.63 (0.137)	5.45 (0.137)	3.69 (0.137	1.14 (0.137)	4.13 (0.060)	2.11 (0.060)	0.67 (0.060)	0.40 (0.011)	N.D.	0.44 (0.217)	N.D.
Mean ± SEM	**11.05± 2.19 (0.117)**	**4.78 ± 0.62 (0.117)**	**3.20 ± 0.30 (0.117)**	**1.22 ± 0.096 (0.117)**	**4.03 ± 0.17 (0.058)**	**1.64 ± 0.38 (0.058)**	**0.60 ± 0.14 (0.058)**	**0.26 ± 0.10 (0.014)**	**N.D.**	**0.60 ± 0.10 (0.14)**	**0.18± 0.20 (0.14)**
Treatment[a]											
RQ4086 (r)	10.69 (0.120)	4.51 (0.120)	1.09 (0.120)	N.D.	4.97 (0.098)	3.57 (0.098)	0.76 (0.098)	0.84 (0.023)	0.35 (0.023)	N.D.	N.D.
(l)	14.01 (0.164)	6.51 (0.164)	0.82 (0.164)	1.98 (0.164)	3.21 (0.059)	1.77 (0.059)	0.56 (0.059)	N.A.	N.A.	0.56 (0.033)	N.D.
RQ4104 (r)	12.07 (0.080)	5.58 (0.080)	3.01 (0.080)	N.D.	3.78 (0.064)	2.83 (0.064)	N.D.	0.59 (0.007)	0.40 (0.007)	0.77 (0.179)	0.44 (0.170)
(l)	8.07 (0.120)	2.54 (0.120)	0.65 (0.120)	N.D.	4.41 (0.071)	2.79 (0.071)	1.00 (0.071)	N.A.	N.A.	0.73 (0.050)	0.52 (0.050)

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; tissue weights are in parentheses (g);[a] Right and left tissues have been designated with (r), (l), respectively; N.D., Not detected; N.A., Tissue not available for analysis.

APPENDIX H. (continued)

Primate ID	Concentrations (ng/tissue) of Lutein, Zeaxanthin, *meso*-Zeaxanthin, and 3'-Oxolutein in Primates' Ocular Tissues (Tissue Weight, g)*										
	Retina				Ciliary Body			Iris		Lens	
	lutein	zea-xanthin	*meso*-zea-xanthin	3'-oxo-lutein	lutein	zeaxanthin	3'-oxo-lutein	lutein	zea-xanthin	lutein	zea-xanthin
Treatment											
RQ4118 (r)	6.54 (0.052)	3.95 (0.052)	1.78 (0.052)	N.D.	1.17 (0.023)	1.61 (0.023)	N.D.	0.48 (0.005)	0.40 (0.005)	0.54 (0.138)	0.27 (0.138)
(l)	3.17 (0.038)	0.92 (0.038)	0.42 (0.038)	N.D.	2.33 (0.044)	1.53 (0.044)	N.D.	0.47 (0.025)	0.36 (0.025)	N.A.	N.A.
RQ4126 (r)	5.08 (0.022)	2.88 (0.022)	1.14 (0.022)	2.52 (0.337)	1.75 (0.072)	1.74 (0.072)	2.26 (0.065)	0.62 (0.008)	0.38 (0.008)	0.33 (0.142)	N.D.
(l)	14.58 (0.164)	64.93 (0.164)	N.A>	1.66 (0.164)	4.70 (0.053)	2.92 (0.053)	1.43 (0.053)	0.34 (0.0024)	0.26 (0.0024)	N.A.	N.A.
RQ4154 (r)	17.50 (0.337)	12.04 (0.337)	3.22 (0.337)	N.D.	14.32 (0.065)	13.43 (0.065)	N.D.	1.49 (0.005)	1.07 (0.005)	0.46 (0.161)	0.32 (0.161)
(l)	23.29 (0.150)	14.88 (0.150)	4.12 (0.150)	3.55 (0.150)	8.36 (0.053)	7.00 (0.053)	1.17 (0.053)	1.38 (0.0073)	1.19 (0.0073)	0.80 (0.045)	0.53 (0.045)
Mean ± SEM	**11.50 ± 2.12 (0.108)**	**5.66 ± 2.02 (0.108)**	**1.75 ± 0.54 (0.108)**	**2.37 ± 0.33 (0.108)**	**4.89 ± 1.66 (0.060)**	**3.92 ± 1.17 (0.060**	**0.80 ± 0.26 (0.060)**	**0.77 ± 0.18 (0.010)**	**0.52 ± 0.15 (0.010)**	**0.56 ± 0.07 (0.107)**	**0.39 ± 0.06 (0.107)**

*Concentrations of *cis*-isomers of lutein and zeaxanthin have been combined with their corresponding all-*trans* isomers; tissue weights are in parentheses (g);[a] Right and left tissues have been designated with (r), (l), respectively; N.D., Not detected; N.A., Tissue not available for analysis.

APPENDIX I. CAROTENOIDS IN THE MAJOR ORGANS AND TISSUES OF L/Z-TREATED PRIMATES AT MONTH 12

Concentrations (μg/g tissue) of lutein, zeaxanthin, 3'-oxolutein, and retinol in the tissues of 5 primates supplemented daily with lutein/zeaxanthin (each at 0.5 mg/kg) for 12 months compared to control primate RQ4079 from the Lutein Supplementation Study.

Tissue	**Primate ID**	**Concentrations (μg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues**			
		lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Liver	Control				
	RQ4079	1.653	0.775	N.D.	11.330
	Treatment				
	RQ4086*	0.982	0.605	N.D.	1.800
	RQ4104	0.279	0.176	N.D.	1.100
	RQ4118	0.855	0.574	N.D.	4.000
	RQ4126*	0.628	0.360	N.D.	3.990
	RQ4154*	0.853	0.678	N.D.	18.012
	Mean ± SEM	**0.719 ± 0.12**	**0.479 ± 0.09**		**5.781 ± 3.11**
Lung	Control				
	RQ4079	0.100	0.510	0.006	0.164
	Treatment				
	RQ4086	0.100	0.699	0.013	0.070
	RQ4104	0.090	51.35	0.009	0.067
	RQ4118	0.033	19.06	0.007	0.057
	RQ4126	0.090	67.28	0.017	0.095
	RQ4154	0.125	126.48	0.023	0.073
	Mean ± SEM	**0.088 ± 0.02**	**0.067±0.02**	**0.014±0.003**	**0.072 ± 0.006**
Colon	Control				
	RQ4079	0.090	0.060	0.036	0.234
	Treatment				
	RQ4086	0.020	0.007	0.003	0.051
	RQ4104	0.528	0.293	0.045	0.617
	RQ4118	0.445	0.345	0.093	0.443
	RQ4126	0.528	0.252	0.055	0.343
	RQ4154	1.232	0.982	0.104	0.461
	Mean ± SEM	**0.550± 0.19**	**0.376 ± 0.16**	**0.060 ± 0.02**	**0.383 ± 0.09**

*Trace amounts of 3'-epilutein detected; [a] Mean value for right and left breast tissues; N.D., Not detected.

APPENDIX I. (continued)

Tissue	Primate ID	Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues			
		lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Kidney	Control				
	RQ4079	0.049	0.027	0.005	1.442
	Treatment				
	RQ4086*	0.078	0.043	0.018	1.363
	RQ4104	0.058	0.044	0.011	1.302
	RQ4118	0.098	0.052	0.034	1.635
	RQ4126	0.744	0.372	0.015	1.135
	RQ4154	0.227	0.206	0.062	1.900
	Mean ± SEM	**0.241 ± 0.13**	**0.144 ± 0.06**	**0.028 ± 0.01**	**1.467 ± 0.13**
Breast	Control				
	RQ4079[a]	0.037	0.020	0.009	0.032
	Treatment				
	RQ4086	0.284	0.196	0.100	0.129
	RQ4104	0.720	0.544	N.D.	0.896
	RQ4118	0.475	0.271	N.D.	0.688
	RQ4126	0.229	0.181	0.057	0.310
	RQ4154	0.491	0.398	0.046	0.481
	Mean ± SEM	**0.440 ± 0.09**	**0.318 ± 0.07**	**0.068 ± 0.02**	**0.501 ± 0.14**
Ovaries	Control				
	RQ4079	0.026	0.017	0.003	0.033
	Treatment				
	RQ4086	0.270	0.095	0.055	0.284
	RQ4104	0.060	0.452	0.016	0.190
	RQ4118	0.308	0.110	0.067	4.270
	RQ4126	0.286	0.137	0.040	1.737
	RQ4154	1.468	1.248	0.268	0.201
	Mean ± SEM	**0.478 ± 0.25**	**0.326 ± 0.23**	**0.089 ±0.05**	**1.336 ± 0.79**

*Trace amounts of 3'-epilutein detected; [a] Mean value for right and left breast tissues; N.D., Not detected.

APPENDIX I. (continued)

Tissue	Primate ID	Concentrations (µg/g tissue) of Lutein, Zeaxanthin, 3'-Oxolutein, and Retinol in Primate Tissues			
		lutein	**zeaxanthin**	**3'-oxolutein**	**retinol**
Spleen	Control				
	RQ4079	0.322	0.138	0.029	0.134
	Treatment				
	RQ4086	0.414	0.285	0.070	0.233
	RQ4104	0.310	0.204	0.044	0.205
	RQ4118	0.315	0.236	0.080	0.326
	RQ4126	0.406	0.297	0.064	0.240
	RQ4154	0.648	0.682	0.165	0.324
	Mean ± SEM	**0.418 ± 0.06**	**0.341 ± 0.09**	**0.085 ± 0.02**	**0.266 ± 0.025**
Cervix	Control				
	RQ4079	0.017	0.013	0.002	0.028
	Treatment				
	RQ4086	0.038	0.017	0.009	0.077
	RQ4104	0.050	0.019	0.006	0.074
	RQ4118	0.039	0.015	0.010	0.136
	RQ4126	0.044	0.027	0.006	0.087
	RQ4154	0.091	0.083	0.020	0.128
	Mean ± SEM	**0.052 ± 0.01**	**0.032 ±0.01**	**0.010 ±0.003**	**0.100 ± 0.014**
Adipose	Control				
	RQ4079	0.163	0.095	0.072	0.147
	Treatment				
	RQ4086	0.848	0.384	N.D.	4.090
	RQ4104	0.311	0.148	N.D.	27.233
	RQ4118	0.014	0.008	N.D.	0.083
	RQ4126	0.113	0.064	N.D.	6.190
	RQ4154	1.855	1.411	N.D.	0.710
	Mean ± SEM	**0.638 ± 0.34**	**0.403± 0.26**		**7.661 ± 5.01**

*Trace amounts of 3'-epilutein detected; [a] Mean value for right and left breast tissues; N.D., Not detected.

APPENDIX J. AGE AT BASELINE AND BODY WEIGHTS AT BASELINE, MONTHS 6, 12, AND 18 FOR L-, Z-, AND L/Z-TREATED PRIMATES

Primate Group	Primate ID	Age at Start (y)	Weight (kg)			
			Baseline	Month 6	Month 12	Month 18
Control	4107*	3.9	3.14	3.84	3.89	3.50
	4142*	3.1	3.34	3.82	3.91	3.92
	4079	3.0	3.28	4.51	4.05	
	Mean±SEM	**3.3±0.3**	**3.25±0.06**	**4.06±0.23**	**3.95±0.05**	**3.71±0.21**
L-Treated	4078*	3.2	3.50	4.37	4.19	4.04
	4189*	3.0	3.84	4.70	4.61	4.57
	4175*	3.3	3.60	4.45	4.61	4.73
	4087	2.3	3.26	3.86	3.88	
	4122	1.9	2.92	3.83	3.82	
	Mean±SEM	**2.7±0.3**	**3.42±0.16**	**4.24±0.17**	**4.22±0.17**	**4.45±0.21**
Z-Treated	4120*	3.1	4.28	5.39	5.29	5.20
	4092*	3.0	4.44	4.13	4.53	4.50
	4094*	3.1	3.96	3.61	4.37	4.30
	4146	2.7	3.96	3.88	4.49	
	4173	3.0	3.94	4.67	5.09	
	Mean±SEM	**3.0±0.1**	**4.12±0.10**	**4.34±0.32**	**4.75±0.18**	**4.67±0.27**
L/Z-Treated	4086	3.0	3.13	3.60	3.50	
	4154	3.3	2.78	3.50	3.84	
	4118	3.0	3.58	4.39	4.29	
	4126	3.0	3.33	3.87	4.04	
	4104	3.3	3.05	3.54	4.05	
	Mean±SEM	**3.1±0.1**	**3.17±0.13**	**3.78±0.17**	**3.94±0.13**	

* Primate sacrificed at month 18; all other primates were sacrificed at month 12.

APPENDIX K. SUMMARY OF THE MEAN CAROTENOID CONCENTRATIONS (MEAN ± SEM) IN THE OCULAR TISSUES OF L-TREATED, Z-TREATED AND L/Z-TREATED PRIMATES AT MONTHS 12 AND 18

Animals	Mean concentrations (ng/tissue) of carotenoids in the ocular tissues of primates								
	Retina, ng/tissue [1] (picomol/tissue)[2]				Ciliary Body, ng/tissue [1]			Iris ng/tissue	Lens ng/tissue
	lutein	zeaxanthin	*meso*-zeaxanthin	3'-oxo-lutein	lutein	zeaxanthin	3'-oxo-lutein	lutein/ zeaxanthin[6]	lutein/ zeaxanthin[6]
Control[3]									
(n = 3)	11.05 ± 2.19[b,c] (19.4 ± 3.8)	4.78 ± 0.62[b] (8.4 ± 1.1)	3.20 ± 0.30[b,c] (5.6 ± 0.5)	1.24 ± 0.10[b,c] (2.2 ± 0.2)	4.03 ± 0.17[a]	1.67 ± 0.38[b]	0.60 ± 0.06[a,b]	0.40 ± 0.01 N.D.	0.60 ± 0.10 0.18 ± 0.20
L-Treated									
Month 12[4] (n = 2)	40.4 ± 1.41[a] (71.0 ± 2.5)	7.56 ± 0.71[b] (13.3 ± 1.3)	4.87 ± 0.70[b] (8.6 ± 1.2)	3.97 ± 0.55[a] (7.0 ± 1.0)	7.12 ± 0.22[a]	1.84 ± 0.58[b]	1.09 ± 0.06[a]	1.68 ± 0.91 N.D.	0.76 ± 0.08 N.D.
Month 18[5] (n = 3)	6.20 ± 0.70[c] (10.9 ± 1.2)	2.34±0.78[b] (4.1±1.4)	1.45 ± 0.44[c] (2.5±0.8)	0.66 ± 0.08[c] (1.2 ± 0.1)	3.64 ± 1.20[a]	1.39 ± 0.36[b]	N.D.	0.59 ± 0.19 N.D.	1.36 ± 0.33 N.D.
Z-Treated									
Month 12[4] (n = 2)	19.2 ± 9.07[b] (33.8 ± 16)	20.4 ± 3.88[a] (35.9 ± 6.8)	7.20 ± 1.37[a] (12.7 ± 2.4)	3.87 ± 0.82[a] (6.8 ± 1.5)	4.97 ± 2.55[a]	12.9 ± 4.28[a]	1.34 ± 0.50[a]	0.26 ± 0.05 0.40 ± 0.01	0.34 ± 0.01 0.37 ± 0.05
Month 18[5] (n = 3)	7.84 ± 1.03[c] (13.8 ± 1.8)	4.45 ± 0.42[b] (7.8 ± 0.7)	1.82 ± 0.45[c] (3.2 ± 0.8)	0.84 ± 0.27[c] (1.5 ± 0.5)	7.18 ± 1.04[a]	3.53 ± 0.62[b]	0.92 ± 0.19[a,b]	0.64 ± 0.01 N.D.	0.63 ± 0.10 N.D.
L/Z-Treated									
Month 12[5] (n = 5)	11.5 ± 2.53[b,c] (20.2 ± 4.5)	5.66 ± 2.02[b] (10.0 ± 3.6)	1.75 ± 0.54[c] (3.1 ± 0.9)	2.37 ± 0.33[b] (4.2 ± 0.6)	4.90 ± 1.66[a]	3.92 ± 1.59[b]	1.17 ± 0.25[a]	0.77 ± 0.18 0.52 ± 0.15	0.56 ± 0.07 0.39 ± 0.06

[1] Means followed with identical superscript letters (a, b, c) within the same column are not significantly different at .05 level of significance; [2] Values for retinas are also expressed in picomol/tissue in parantheses; [3]Average of the values for 1 control animal sacrificed after 12 months and 2 control animals sacrificed after 18 months; [4] Only one eye from each animal was available for analysis as the other eye was used for histopathology; [5] Includes average values for the left and the right eye; [6]combined values for zeaxanthin and *meso*-zeaxanthin; N.D., Not detected.

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