THE EFFECTS OF CARROT CAROTENOIDS ON VISUAL FUNCTION IN

LONG-HOUR COMPUTER USERS: A PILOT STUDY

by

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Abstract

Although carotenoids are essential for visual function, their potential beneficial role in Computer Vision Syndrome (CVS) remains to be elucidated. By providing carrot powder rich in α- and β-carotene this study examined whether carrot carotenoids can influence retinal function in long-hour computer users. A double-blind, placebo-controlled, repeated measures pilot trial, consisting of male and female participants (n=19, ages 20-65) with CVS, were randomly assigned to two supplementation groups; control (15g cream of wheat powder) or carrot enriched (15g carrot powder, 33% of vitamin A RDA for adults) in the form of an isocaloric pudding and yogurt, every day for 4 weeks. Retinal function was assessed with the electroretinogram (ERG) at PRE (week 0) and POST (week 4) time points. Plasma oxidative stress markers, lipids, and carotenoid/retinoid levels were assessed using ELISA, auto-analyzer, and UPLC, respectively, at PRE, DURING (week 2), and POST. Self-perceived vision status was assessed using the Ocular Surface Disease Index questionnaire. Carrot supplementation marginally improved ($P<0.1$) photopic b-wave amplitudes, without reaching statistical significance, representing cone-driven phototransduction, in 75% of total retinas, indicating retinal sensitivity to dietary nutrients. Carrot supplementation significantly increased plasma retinol and β-carotene levels ($P<0.02$), however, were not associated with CVS symptom improvement. HDL cholesterol significantly increased, whilst LDL cholesterol exhibited a diet, lowering trend ($P<0.09$). Plasma F2-isoprostanes displayed a trending reduction ($P<0.1$) compared to the control group illustrating the anti-oxidant potential of the carrot. Improvements seen in cone-driven inner retinal responses, along with increased plasma carotenoid/retinoid levels and beneficial lipid and oxidative stress changes, indicate that minimal supplementation of carotenoids at 33% of the vitamin A RDA by carrot powder can be recommended as a novel nutritional therapy for healthy, chronic computer users. A larger sample size test is warranted to obtain robust results.

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List of Abbreviations

A2E: N-retinylidene-N-retinylethanolamine

ABCA1:   ATP-binding cassette transporter ABCA1

AMD: age related macular degeneration

AOA: American Optometric Association

ARAT: acyl-CoA-retinol acyltransferase

BCMOI: β,β-carotene 15,15'-monooxygenase

BCDOII: β,β-carotene 9',10'-dioxygenase

BHT: butylated hydroxytoluene

BMI: body mass index

CFIA: Canadian Food Inspection Agency

CRALBP: cellular retinaldehyde-binding protein

CRBPI: cellular retinol-binding protein (CRBPI & CRBPII)

CV: coefficient of variation

CVD: cardiovascular disease

CVS: computer vision syndrome

DHA: docosahexaenoic acid

DRI: dietary reference intakes

EAR: estimated average requirement

ECG: electrocardiography

ELISA: enzyme linked immunosorbent assay

ERG: electroretinography

GCL: ganglion cell layer

HDL: high-density lipoprotein

HIV:	human immunodeficiency virus
HPLC:	high performance liquid chromatography
ILM:	inner limiting membrane
INL:	inner nuclear layer
IPL:	inner plexiform layer
IRBP:	inter-photoreceptor retinol binding protein
IU:	international units
LDL:	low-density lipoprotein
LPL:	lipoprotein lipase
LRAT:	lecithin-retinol acyltransferase
MDA:	malondialdehyde
MPOD:	macular pigment optical density
NADPH:	nicotinamide adenine dinucleotide phosphate
NFL:	nerve fiber layer
NS:	not significant
OLM:	outer limiting membrane
ON:	outer nuclear
ONL:	outer nuclear layer
OP:	oscillatory potential
OPL:	outer plexiform layer
OSDI:	Ocular Surface Disease Index questionnaire
RAE:	retinol activity equivalents
RBP:	retinol binding protein
RDA:	recommended dietary allowance
RDH:	retinol dehydrogenase (RDH 5, RDH 10, RDH 11)

RE: retinyl ester

REH: retinyl ester hydrolase

ROS: reactive oxygen species

RPE: retinal pigment epithelium

sc cd·sec/m^2:   scotopic candela sec/meter square

SCFA: short-chain fatty acids

SD: standard deviation

SEM: standard error of the mean

SNP: single nucleotide polymorphism

SOD: superoxide dismutase

SR-BI: scavenger receptor class B member 1

STRA6:   stimulated by retinoic acid 6 gene

STZ: streptozotocin

TC: total cholesterol

TG: triacylglyceride

TTR: transthyretin

UNICEF: The United Nations Children's Fund

UPLC-PDA: ultra-performance liquid chromatography-photodiode array

VDT: video display terminal

VLDL: very low density lipoprotein

WHO: World Health Organization

Chapter I: INTRODUCTION

The computer has become a significant and essential part of societies' daily life placing increased visual demands on the eyes, ultimately contributing to ocular discomforts. The main visual symptoms associated with the use of computers are eyestrain, irritation, tired eyes, redness, blurred vision and double vision (Blehm et al., 2005). These vision related discomforts are collectively coined as Computer Vision Syndrome (CVS). The American Optometric Association (AOA) (2014) defines CVS as "the complex of eye and vision problems related to near work, which are experienced during or related to computer use." In Canada in 2001, 8.3 million people used the computer at work, and of these computer users it is estimated that 70-75% experience some sort of discomfort related to computer use (Statistics Canada, 2010), which may potentially hinder users quality of life and productivity. Suggestions have been made to reduce visual discomfort such as taking regular breaks, altering the location of the computer, changing the lighting and reflection from the screen, and using eye drops, however, these treatment options do not provide users with complete reprieve of their symptoms nor prevent them from reoccurring. These symptoms remain a major challenge, indicating the need for some therapeutic strategies, such as nutrition.

The carrot has been a well-known health food for the eyes for centuries. It is a leading dietary vegetable around the world and one of the richest sources of carotenoids (β-carotene, α-carotene and lutein). Carotenoids act as antioxidants and precursors of vitamin A, an essential component of the visual cycle. The effects of purified vitamin A, β-carotene and lutein alone have been extensively studied in connection with visual health, but the therapeutic value of the whole carrot, containing several carotenoids and other nutrients, has not been tested. A recent animal study conducted in our laboratory, showed significant improvements in visual function (rod and cone cell function) in

healthy animals following supplementation of carrot powder (McClinton et al., 2014). Thus, it is necessary to know whether similar beneficial results can be seen in individuals with CVS. The results of this study will contribute to the development of simple therapeutic solutions for CVS and possibly other vision related syndromes.

The following chapter provides a basic overview of carotenoid and vitamin A metabolism, their role in visual function, current vitamin A dietary recommendations, and current CVS knowledge.

Carotenoid and vitamin A metabolism

Classifications

Vitamin A must be obtained from the diet either as preformed vitamin A compounds, such as retinol and retinyl esters (RE), or from provitamin A carotenoids, β-carotene, α-carotene and β-cryptoxanthin, which are then converted to vitamin A derivatives in the body (Fig. 1-1 & 1-2). Generally, preformed vitamin A compounds are obtained from animal-derived food sources such as liver and dairy products (Ball, 2006). Comparatively, carotenoid precursors are obtained from plant-derived food sources such as green leafy vegetables and orange fruits and vegetables (Ball, 2006).

Only six, (β-carotene, β-cryptoxanthin, α-carotene, lycopene, lutein and zeaxanthin) of the approximate 600 carotenoids isolated from natural sources, comprise around 95% of the total blood carotenoids in humans (Maiani et al., 2009) (Fig. 1-2).

Absorption and metabolism

Vitamin A is an essential nutrient for vision, cellular differentiation, growth, reproduction, bone development and immunological function. Vitamin A acts as a precursor to two molecules; 11-*cis*-retinal, a core component of rhodopsin, a visual pigment protein found in photoreceptor cells, and all-*trans*-retinoic acid, a hormone-like

ligand responsible for gene expression in differentiation of a variety of cell types (O'Byrne and Blaner, 2013).
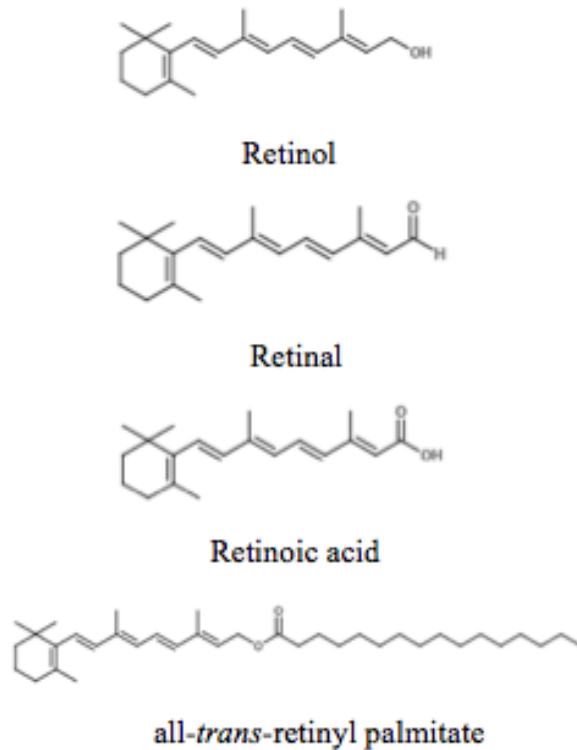
Figure 1-1: Chemical structures of the major vitamin A derivatives present in human tissues.

Following ingestion of dietary vitamin A, there are five crucial steps involved in the metabolism of retinoids and carotenoids, which will be discussed in detail in the upcoming review; 1) release from food matrix 2) solubilization into mixed micelles 3) uptake by the intestinal mucosal cells 4) incorporation into chylomicrons and 5) secretion into the lymph (During et al., 2002).
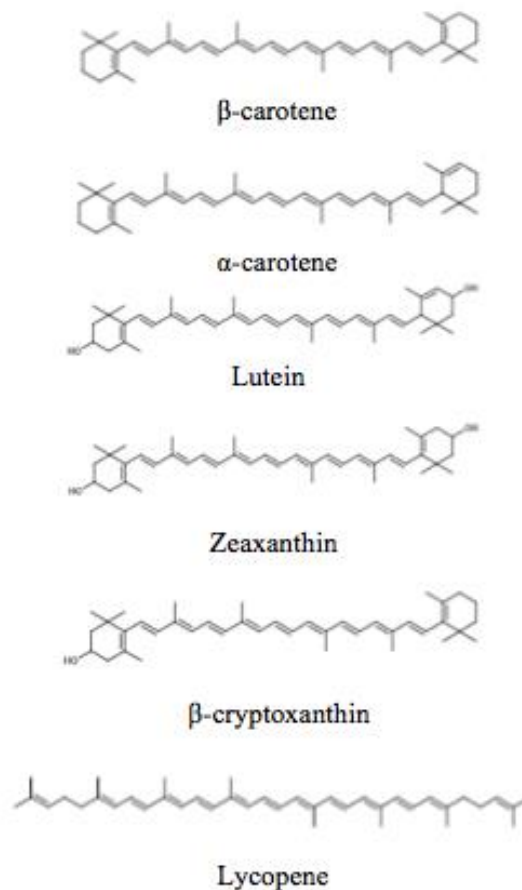
Figure 1-2: Chemical structures of the major carotenoids found in fruits and vegetables, which are present in significant levels in human plasma.

Retinoid metabolism

The two most abundant retinoid forms found in the diet are retinol and RE (Fig. 1-1). Upon consumption, retinoids must be hydrolyzed from the proteins and fatty acids that they are bound to by action of pepsin in the stomach, and proteolytic enzymes in the small intestine, respectively (Gropper et al., 2009). Following dissolution of freed REs and carotenoids within lipid droplets, they are then incorporated into mixed micelles through the assistance of bile salts and pancreatic lipase, further entering the enterocyte by passive diffusion (Parker, 1996). Parker (1996) suggests rate of diffusion into the enterocyte is determined by the concentration gradient between the micelle and the

plasma membrane of the enterocyte. Conversely, dietary retinol can be directly taken up by mucosal cells, however, dietary REs cannot enter the enterocyte without the action of luminal retinyl ester hydrolase (REH) to yield free retinol. Dietary REs in the intestinal lumen can be hydrolyzed by enzymes such as pancreatic triglyceride (TG) lipase, cholesterol ester hydrolase, and intestinal brush border phospholipase B, to form unesterified retinol, the form absorbed by the intestinal mucosal cell (During and Harrison, 2007) (Fig. 1-3).

Carotenoid metabolism

Provitamin A carotenoids may be converted to retinal and absorbed, or carotenoids may be absorbed as is (O'Byrne and Blaner, 2013). Unlike retinoids, which can be passively absorbed into the enterocyte, recent evidence suggests that absorption of carotenoids occurs by a facilitated mechanism instead (During et al., 2002). Using the CaCo-2 cell culture line, which mimics the *in vivo* intestinal absorption of carotenoids, During et al. (2002) suggest that intestinal transport of β-carotene is highly regulated by the participation of transporters and intracellular receptors (scavenger receptor class BI, SR-BI), which preferentially incorporate all-*trans*-β-carotene versus the *cis* isomers into chylomicrons.

Once inside the enterocyte, retinol and carotenoids are released from micelles via dissolution. A series of enzymatic reactions take place inside the enterocyte through the action of cellular retinol-binding protein I & II (CRBPI & CRBPII), lecithin : retinol acyltransferase (LRAT), acyl-CoA-retinol acyltransferase (ARAT), and ATP-binding cassette A1 (ABCA1), which incorporate REs and carotenoids into chylomicrons (During and Harrison, 2007; MacDonald and Ong, 1988; Batten et al., 2004).
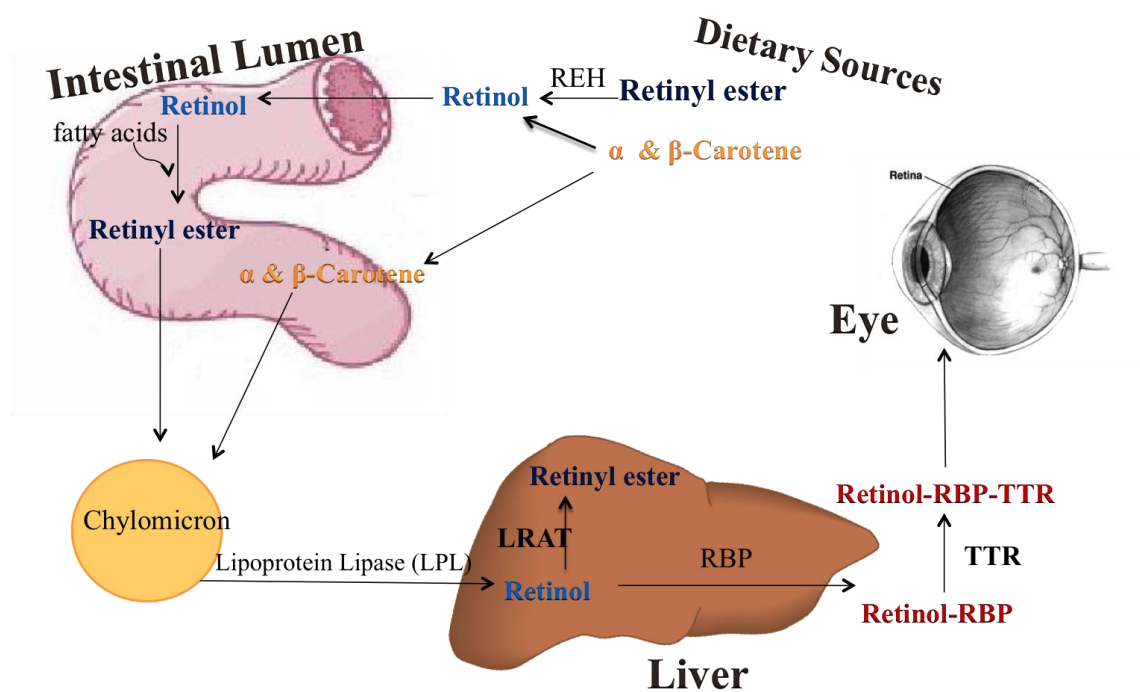
Figure 1-3: Basic vitamin and carotenoid transportation and conversion in the body. LRAT, lecithin : retinol acyltransferase; RBP, retinol binding protein; REH, retinyl ester hydrolase; TTR, transthyretin.

Absorbed provitamin A carotenoids can undergo three metabolic processes converting them to retinoids prior to entering the portal blood. The two major sites of β-carotene conversion in humans are the intestine and the liver (Maiani et al., 2009). The first and major reaction is the central cleavage reaction; the enzyme β,β-carotene 15',15'-monooxygenase (BCMOI) splits the β-carotene molecule at the central double bond to yield two retinal molecules, which can further be reduced to retinol by retinol reductase, and esterified (Castenmiller and West, 1998). The second reaction being non-central or eccentric cleavage reaction; the enzyme β,β-carotene 9',10-dioxygenase (BCDOII) cleaves the double bond at the 9' position, producing β-apo-carotenal which can be converted to 2 molecules of retinal or to β-apo-carotenoic acids and further to retinoic acid (Tang and Russell, 2009). Wolf (1995) elicited evidence supporting the mutual

existence of both central and eccentric cleavage reactions. Thirdly, random cleavage of β-carotene can occur by free radicals that are produced by enzymes such as lipoxygenase (Yeum and Russell, 2002). In a study conducted by Yeum and colleagues (2000), they demonstrated that both central and random cleavage of β-carotene can occur in the post-mitochondrial part of rat intestine depending on the presence of α-tocopherol. Conversely, instead of being metabolized into smaller molecules, a limited amount of dietary carotenoids can be absorbed intact (Goodman, 1984).

Many animal and human studies have been conducted examining the interactions among various carotenoids, with most studies eliciting mixed results (Kostic et al., 1995; O'Neill and Thurnham, 1998; Paetau et al., 1997; van den Berg et al., 1998). Interactions between carotenoids can occur at any of the various stages throughout absorption. Studies conducted have shown supplementation of β-carotene inhibited lutein (Kostic et al., 1995) and canthaxanthin (O'Neill and Thurnham, 1998; Paetau et al., 1997) absorption. Comparatively, other investigators have shown that a high dose supplementation of both lycopene and β-carotene decreased the bioavailability of both compounds (van den Berg et al., 1998). Hoppe and colleagues (2003) demonstrated that both synthetic and natural lycopene sources have no interaction with circulating β-carotene, β-cryptoxanthin, α-carotene, lutein or zeaxanthin. Collectively, this research suggests that these carotenoids could exhibit a similar absorption mechanism. It is evident that further research is needed to clarify the interactions between carotenoids, especially in terms of the multiple carotenoids that are found in whole food matrices.

Once chylomicrons from the intestinal mucosa enter the bloodstream via lymph, they are degraded by lipoprotein lipase (LPL) releasing its contents including glycerol, fatty acids and retinol or carotenoids. These released retinoids and carotenoids are then rapidly absorbed by the liver hepatocyte (Yeum and Russell, 2002) (Fig. 1-3). Following

dissolution, chylomicron remnants are taken up by the liver via receptor-mediated endocytosis (Goodman, 1984). REs are then hydrolyzed by REHs to yield free retinol. Retinol may then either be stored or bound to retinol binding protein (RBP) and transthyretin (TTR) for transport to tissues depending on vitamin A status. Under vitamin A necessitated conditions, retinol is transported to tissues with the help of RBP and TTR. A transmembrane protein, stimulated by retinoic acid gene 6 (STRA6), acts as a RBP receptor within target tissues, needed for cellular uptake (Ross et al., 2014). Interestingly, high levels of STRA6 are present within the retinal pigment epithelium

Conversely, under vitamin A sufficient levels, hepatic stellate cells act as the primary storage site of vitamin A, as confirmed by Blomhoff et al. (1982). The authors identified that following injection of radiolabeled REs to vitamin A deficient rats the majority of the REs were located within the plasma. In comparison, radiolabeled vitamin A provided to vitamin A sufficient rats primarily appeared in hepatic stellate cells (Blomhoff et al., 1982). Liver stores account for over 70% of total body stores that fluctuate in accordance with vitamin A status (Blaner et al., 2009), with as much as 90-95% of stores in the form of REs (Gropper et al., 2009). Retinyl palmitate represents the predominant RE within stellate cells constituting 70-75% of REs, followed by retinyl stearate, retinyl oleate and retinyl lineolate, respectively (Schreiber et al., 2012). Hepatic stellate cells possess the optimal storage environment due to their high levels of LRAT enzymes and CRBP (Schreiber et al., 2012). The mobilization of stored vitamin A is a highly regulated process, indicating that fasting plasma retinol levels are maintained between 2-4µmol/L in humans, unless deficient, due to homeostatic mechanisms (O'Byrne and Blaner, 2013). Normal vitamin A serum concentrations range from 0.3 to 1.2 mg/L (Aleman et al., 2013). Carotenoids found in the blood are transported as part of lipoproteins. Polar carotenoids (lutein and zeaxanthin) are carried mainly on high-density lipoproteins (HDLs) (53%), whereas low-density lipoproteins (LDLs) carry 58-73% of β-

carotene, α-carotene and lycopene (Gropper et al., 2009). Numerous tissues including, but not limited to, adrenal glands, the epidermis, sex organs, and the small intestine, uptake vitamin A in the form of REs found circulating in chylomicrons in order to function (Lindqvist et al., 2005). However, no REs are taken up by the retina, which will be discussed further in the upcoming review.

Following circulation throughout the body, the kidneys are responsible for the excretion of both retinoids and RBPs. The receptor protein, megalin, located on the apical surface of the renal proximal tubule cells, facilitates in the recovery and recycling process of retinol and RBP (Christensen et al., 1999). Since 85-90% of circulating RBP is bound with retinol, this mediated uptake of RBP allows for minimal loss (~10%) of retinol in the urine under ideal physiological conditions (Christensen et al., 1999). Quantitatively, of the 10% of vitamin A that is not absorbed, 20% is excreted in the feces through the action of bile, 17% appears in the urine, 3% is released as carbon dioxide, and 50% is stored in the liver (Olson et al., 1996). Dueker and colleagues (2000) conducted a labeled isotope experiment highlighting that urinary excretion is not the major route of excretion for intact β-carotene. After administration of a 15 mg dose of β-carotene, subjects excreted 83% of the dose in their feces (Ball, 2006).

Vitamin A deficiency

The World Health Organization (WHO) estimates around 250 million children are vitamin A deficient and of these children, approximately 250,000 to 500,000 become blind every year (WHO, 2013). Vitamin A deficiency is a public health concern for young children and pregnant women in less industrialized parts of the world, especially in Africa and South-East Asia (WHO, 2013). Individuals with vitamin A deficiency are more prone to experience diarrhea, measles, malaria and other diseases (Salazar-Lindo et al., 1993; Hussey and Klein, 1990; Samba et al., 1990; Filteau et al., 1993). Suboptimal

vitamin A intake has also been observed in developed countries. According to Health Canada (2012), more than 35% of Canadians 19 years old and over were consuming less vitamin A than the estimated average requirement (EAR), a value that meets requirements of 50% of the population. This data is of concern for women of childbearing age due to the fact that pregnancy can further deplete vitamin A levels, leading to severe deficiency symptoms.

Vitamin A deficiencies can occur as primary or secondary deficiencies. Primary deficiencies are the most common type of deficiency and occur as a result of a lack of dietary intake in vitamin A rich foods such as liver, eggs, carrots, spinach and broccoli. Secondary deficiencies are less common and are a result of malabsorption of vitamin A in the gastrointestinal tract. Specific examples of diseases that affect the vitamin A status of an individual include Crohn's disease, celiac disease, various infections or impaired biliary or pancreatic secretion (Ross et al., 2014). Vitamin A deficiency symptoms present in these conditions usually manifest within the eye with xerophthalmia being the first symptom to arise followed by xerosis of the eye (Ross et al., 2014). Other extraocular symptoms include perifollicular hyperkeratosis, impaired taste, and infertility (Ross et al., 2014).

Biochemical tests such as plasma retinol concentration and concentration of vitamin A or RBP in breast milk and tear fluid can be used as an indicator of vitamin A deficiency (Ross et al., 2014). Typically, based on previous studies, a plasma retinol concentration of less than 0.35, 0.70 and 1.05 μmol/L is classified as severely deficient, marginally deficient, and subclinical low vitamin A status, respectively (Ross et al., 2014). In animal models, the first sign of deficiency is loss of appetite; in humans, xerophthalmia is an early sign of deficiency after the diet has been deficient for an extended period time (Gropper et al., 2009). The WHO emphasizes the importance of

adequate vitamin A intake for children in speculation that it will reduce young-child

mortality rates by 23-34% (Sommer, 2008; McLaren, 2004; Beaton et al., 1993). A study

conducted on pregnant Nepali women supplemented with a low dose of vitamin A or β-

carotene observed a reduction in pregnancy-related mortality rates (West, 2002).

Those at a higher risk for developing vitamin A deficiency are young children and

pregnant and lactating women. As previously emphasized, vitamin A encompasses a vast

array of functions within the body. Some of the symptoms that occur when deficiency

arises may be temporary or permanent. Visual deterioration, specifically xerophthalmia

and 'night-blindness', is one of the most detrimental effects that ensue from vitamin A

deficiency. Since vitamin A has roles in both the retina and epithelial cells, vitamin A

deficiency can be seen through multiple presentations in the eye (Lien and Hammond,

2011). When levels of vitamin A are low, production of 11-*cis*-retinal is hindered, which

is the main substrate of rhodopsin and it also plays a key role in the transduction of the

visual cycle (Sommer, 2008). McBain and colleagues (2007) have reported the

detrimental effects of vitamin A deficiency on visual function in humans measured using

electroretinogram (ERG), as well as post-treatment improvements following vitamin A

injections. Vitamin A deficiency severely affects rod function and cone implicit time, the

amount of time it takes for cone cells to respond to photic stimulus, however, only 3 days

post injection nearly normalized rod ERG function was achieved, with complete recovery

seen at 12 days post injection (McBain et al., 2007). This research substantiates the vital

role vitamin A plays in visual function. Other ocular diseases associated with deficiency

include xerosis, dryness of the bulbar conjunctiva, due to a lack of mucous being

produced, and keratinized squamous epithelial cells (Bitot's spots) (Ross et al., 2014).

Dixon and Goodman (1987) elicited the response of both normal and retinol-

deficient rat hepatocytes with regard to RBP stores, TTR and retinol. Since RBP is the

major retinol transport protein, the authors (1987) illustrated that in retinol-deficient hepatocytes, there was an increased amount of RBP stores in comparison to the isolated hepatocytes that were not deficient. Of interest, the addition of retinol to the retinol-depleted hepatocytes greatly fueled RBP secretion from these cells as a result of a concentration dependent, saturable process (Dixon and Goodman, 1987). Maximal saturation of RBP secretion was observed at a retinol concentration of 0.3 µg/mol (Dixon and Goodman, 1987). Administration of retinol to normal hepatocytes did not influence RBP secretion or metabolism. Infection-associated hyporetinemia is hypothesized to produce an acute retinol deficiency via negative action on acute phase proteins, such as mRNA levels of RBP and TTR, by pro-inflammatory cytokines (Rosales et al., 1996). It is thus critical to ensure vitamin A status is addressed and resolved in patients presenting with such infection.

A partnership between the WHO and the United Nations Children's Fund (UNICEF) has launched a program called the Vitamin A Global Initiative in 2013, which provides supplementation and immunization to underdeveloped countries. Dosages are administered every 4 to 6 months annually, with optimal doses for various age groups ranging from 100,000 to 200,000 IU (30,000 to 60,000 retinol activity equivalents (RAE)) (The WHO, 2013). Effort has been made to increase awareness and intake of dietary carotenoids, however, supplementation is the single most cost-effective intervention to prevent blindness.

Vitamin A toxicity

Increasing interest and availability of fortified foods and supplements in developed nations has led to a large percentage of the population with a preformed vitamin A intake that is higher than recommended (Allen and Haskell, 2002). Acute toxicity of preformed vitamin A is less of an issue in comparison to chronic toxicity.

Penniston and Tanumihardjo (2006) report chronic toxicity results following ingestion of high amounts of preformed vitamin A for months or years. Daily intakes exceeding 25,000 IU for more than 6 years and intakes exceeding 100,000 IU for more than 6 months are considered toxic levels, however, large interindividual variability exists when determining the minimum dose to produce toxicity (Penniston and Tanumihardjo, 2006). Toxic manifestations of vitamin A may also be enhanced during pregnancy with teratogenicity proceeding daily doses of 40,000 IU of vitamin A in the first trimester of pregnancy (Martinez-Frias & Salvador, 1990). Elevated levels of REs greater than 10% of the total circulating vitamin A is used as a biomarker for chronic hypervitaminosis A (Penniston and Tanumihardjo, 2006). Side effects of toxicity are variable between individuals and dose dependent with symptoms including vomiting, severe headaches, dizziness, blurred vision and osteoporosis (Ball, 2004). Comparatively, high intake of provitamin A compounds does not result in hypervitaminosis A due to tightly regulated conversion processes (Olson, 1996). However, hypercarotenemia may ensue following chronic consumption of 20mg or more of carotenoids, resulting in a yellowing of the skin (Blomhoff, 2001).

Dietary recommendations

The recommended dietary allowance (RDA) for preformed vitamin A based on the Dietary Reference Intakes (DRI) is 900 µg RAE/day for men and 700 µg RAE/day for women aged 14 to 70+ years (Institute of Medicine, 2000). Preformed vitamin A RDA is set at 400µg–1300µg per day, depending on the present life-cycle stage (Institute of Medicine, 2000). Separate RDAs specific for various life stages such as, during pregnancy or lactation when needs may be varied, are provided.

Retinol equivalency conversion factor has been implemented to determine the vitamin A (retinol) potential of provitamin A carotenoids due to the varying content

within foods (Ross et al., 2014). Currently, 1 RAE is equal to 1 µg of retinol, 12 µg β-carotene, 24 µg α-carotene, 24 µg β-cryptoxanthin and 3.33 IU retinol (Health Canada, 2010).

The tolerable upper limit for preformed vitamin A is set at 3000 µg RAE/day for the adult age group (Institute of Medicine, 2000).

Retina anatomy

During development, the retina is formed from an invagination of the diencephalon that becomes encased by protective layers of the eyeball behind the pupil and lens (Kolb, 1994). Due to the embryonic nature of the retina arising from the neuroectoderm, the retina is actually comprised of neural tissues. The retina is composed of light-sensitive nervous tissue lining the posterior half of the eye ball which when stimulated, elicits an excitatory cascade of synaptic events sending chemical and mechanical signals to the visual center of the brain. The retina is comprised of 10 layers; retinal pigment epithelium (RPE), photoreceptor cell layer, outer limiting membrane (OLM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL) and the inner limiting membrane (ILM) (Fig. 1-4).

The outer nuclear layer contains cell bodies of rods and cones. The inner nuclear layer contains the cell bodies of the bipolar, horizontal and amacrine cells. The ganglion cell layer contains ganglion cell bodies and some amacrine cells. Regions of neuropil separate the nerve cell layers. The first region of neuropil is the outer plexiform layer. The OPL is where the contact occurs between the photoreceptors and horizontal and bipolar cells, producing the first stage in image processing (Margalit and Sadda, 2003). The second region of neuropil is the inner plexiform layer. The IPL acts to transmit information from the bipolar cells to ganglion cells, whose primary function is to relay

the image information to the visual centers in the brain (Margalit and Sadda, 2003).

Within the photoreceptor layer in the retina exists two types of photoreceptors; rod and

cone cells. In the photoreceptor layer there are about 100 million rods, which help

contrast and function in dimly lit lighting and 3 million cones, which perceive colors and

help function in bright lighting (Ball, 2006).
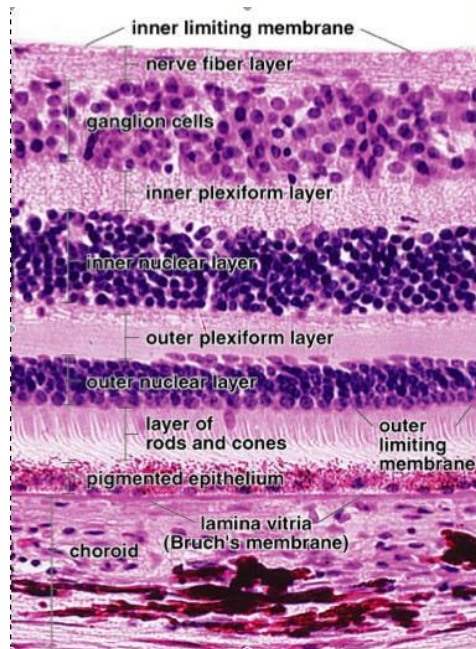
Figure 1-4: A cross-sectional image of the normal human retinal layers (Ross and
Pawlina, 2011). Permission granted 08/11/14 by Caren Erlichman Wolters Kluwer, 2700
Lake Cook Road, Riverwoods, IL, USA.

Retinal pigment epithelium

The RPE is a crucial supporting structure to the retina. This monolayer of

pigmented cells found between the photoreceptor cells and choroid, embodies numerous

functions. Such functions include transport of nutrients, ions and water, secretion of

integral retinal factors, absorption of light energy for protection against photooxidation,

and reisomerization of all-*trans*-retinal to 11-*cis*-retinal, an essential compound in the

visual cycle needed to maintain photoreceptor excitability (Simó et al., 2010; Strauss, 2005).

The retina is subjected to direct and frequent light exposure, increasing its susceptibility to lipid photooxidation, and a high amount of oxygen consumption leading to a high rate of reactive oxygen species (ROS), all of which can produce detrimental effects to retinal cells (Simó et al., 2010). Thus, the RPE acts as an essential tissue within the retina to counterbalance the extensive oxidative stress present via action of three main lines of defense. The first line of defense is absorption and filtration of light. To produce these results, the RPE contains a complex array of pigments that are each specialized at absorbing and defending against various wavelengths (Strauss, 2005). Melanin, lutein/zeaxanthin, and lipofuscin are the three main pigments present in the RPE. Melanin, via action of melanosomes, is responsible for general light absorption, with additional aid from photoreceptors in absorbing general light (Strauss, 2005). Xanthophylls, lutein and zeaxanthin, act to absorb incoming blue light; blue light is believed to be the type of light most detrimental to RPE cells as it permits lipid photooxidation (Carpentier et al., 2009). These three pigments, melanin, lutein, and zeaxanthin, only account for 60% of light energy absorption (Strauss, 2005). This indicates the presence of other pigments, which remain to be fully elucidated. Lipofuscin is one of these pigments that may act in a beneficial nature to the retina. Lipofuscin is able to auto-fluoresce when excited by short wavelength light (Beatty et al., 2000). Lipofuscin accumulates in the RPE throughout life; however, in the older eye lipofuscin appears to attain a toxic concentration, damaging the RPE (Strauss, 2005). The second line of defense is due to enzymatic antioxidants. Antioxidants help protect the retina against oxidative stress by binding with free radicals and peroxides. The RPE contains high amounts of superoxide dismutase (SOD) and catalase (Frank et al., 1999). It has been shown that in response to increased levels of oxygen, cells increase SOD production

in order to counteract the oxygen species (Hassan, 1988). Additionally, catalase activity has shown reductions in oxidative damage due to peroxides (Liles et al., 1991). A study conducted by Liles and colleagues (1991) found that SOD activity showed no significant correlations with aging or macular degeneration, however, catalase decreased with both age and macular degeneration in the RPE. The third line of defense is due to non-enzymatic antioxidants. The RPE accumulates antioxidants such as carotenoids, lutein, zeaxanthin, and ascorbate, which help to combat oxidative stress. Numerous studies have elucidated the beneficial nature of lutein and zeaxanthin against age-related macular degeneration and cataracts by directly interacting with reactive oxygen intermediates to yield a harmless product and prevent further free radical damage. Ascorbate, part of the vitamin C class found in the retina, is also associated with protective effects in part due to the preservation of docosahexaenoic acid (DHA) in the outer rod segment (Beatty et al., 2000).

Electroretinogram

In the presence of light stimuli, photoreceptor cells and other retinal cells are activated producing digression in the interlayer currents, contributing to the changes of retinal electrical potential that can be read on the corneal surface. The full-field ERG is an electrophysiological test that utilizes these retinal potential changes to measure the summed responses of the retinal cells when evoked by light stimuli. This assessment provides an objective measurement of the retina response. A thin, wire electrode is positioned on the cornea and connected to the computer in order to record the changes in electrical potential. Additionally, a reference electrode is placed on the forehead and a ground electrode is attached to the right earlobe with conductive gel, all of which are connected to the amplifier and computer (Fig. 1-5). Full pupil dilation is warranted for a more accurate and standardized response across the retina via action of 1% tropacamide. Following a minimum of 20 min dark adaptation, the patient places their head within the

Ganzfeld dome where the ERG will emit light flashes and record the data obtained (Marmor et al., 2009).
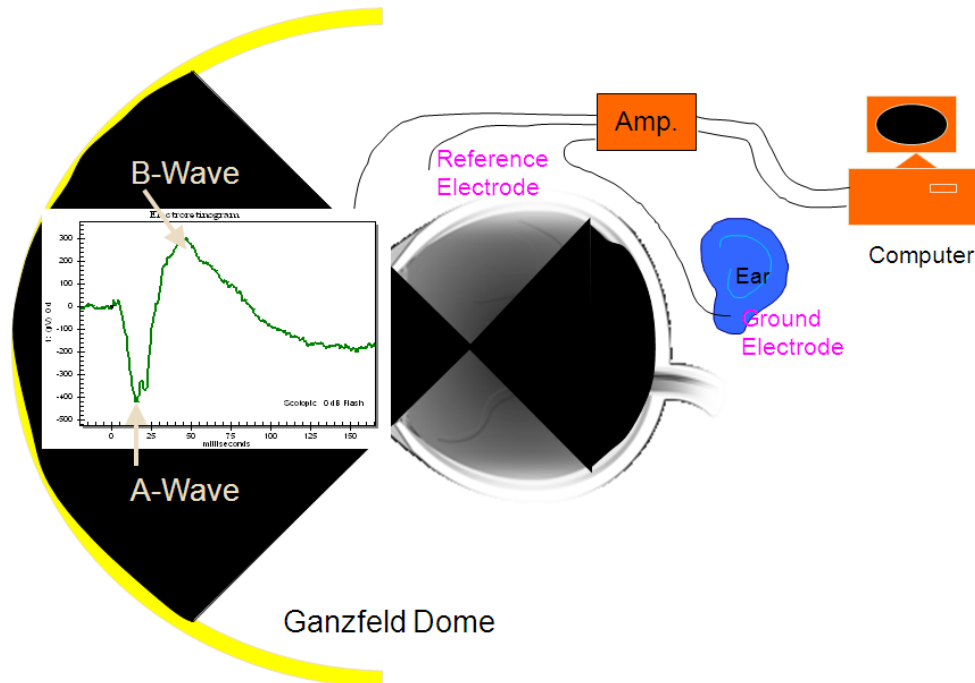
Figure 1-5: Schematic overview of electroretinogram. Diagram adapted from LKC Technologies Inc. *Functional Testing of the Eye* training materials. Permission granted 08/05/12 by Daniel Albano LKC Technologies, Inc., 2 Professional Drive, Gaithersburg, USA.

The ERG data is divided bilaterally into three major components: a-wave, b-wave and oscillatory potentials (OP) (Marmor et al., 2009). Two indices are assessed from the ERG waves: the amplitude of each wave and the time from the beginning of the light stimuli to the trough of the a-wave or peak of the b-wave. These times measured are referred to as 'implicit times' and indicate how long from the onset of the flash did it take the cells to respond. The a-wave is measured from the baseline point to the most negative trough. The a-wave is indicative of the response elicited from the photoreceptor cells. The b-wave is measured from the trough of the a-wave through to the highest peak. In contrast, the b-wave response is an indicator of the health of the inner retinal layers

including the Müller cells and outer nuclear (ON) bipolar cells. OPs are measured on the ascending part of the b-wave peak, which indicate the activity of the amacrine cells found in the inner retina.

Full-field ERG measurements are obtained using a combination of various responses including, mixed scotopic, photopic and photopic flicker. The scotopic, or dark-adapted response, is measured following dark-adaptation of the patient in order to assess rod function. Photopic, or light adapted response, is measured during exposure to a 3.0 cd·sec·m^{-2} background white light to assess cone-driven intensity responses. The photopic flicker response is also indicative of the cone cells activity. By using high frequency flicker in the presence of bright illumination to blanch the rod cells rendering them inactive, the resulting recorded amplitude is indicative of cone cell activity (An et al., 2012)

Westall and colleagues (2001) have established a database of normal ranges for amplitude and implicit times of ERG responses. In normal vision, human a-wave amplitudes and implicit times follow a distribution of 279 ± 46.4 µV (mean ± SD) and 20.4 ± 0.8 msec, respectively. Human b-wave amplitudes and implicit times follow a distribution of 547 ± 103.3 µV and 43.11 ± 3.5 msec, respectively. Although a normative reference range can be generated, there are no 'standard' amplitudes and implicit times for ERG responses. This is due to variations in equipment, recording programs, and electrode types. Laboratories are encouraged to produce their own database of 'normal' values, appropriate for the age and health of individuals commonly assessed (Whatham et al., 2013).

Carotenoids and Vitamin A in visual function

Rod visual cycle

Prior to vitamin A being a useful compound for the RPE, metabolic steps must occur in order for the visual cycle to transpire. All-*trans* retinol, secreted from body and liver stores, is complexed with TTR and RBP where it is mobilized and transported through the blood and into the RPE. RBP acts to solubilize, protect and detoxify retinoids in the intracellular and extracellular environment (O'Byrne and Blaner, 2013). The process by which carotenoids are taken up into RPE is still largely unknown, however, studies have suggested the involvement of SR-BI and the multi-transmembrane domain protein STRA6 (During et al., 2008; Isken et al., 2008, respectively). Targeted gene knockout of carotenoid enzyme, BCMOI, has shown the importance of β-carotene in contribution to the retinal content in the eye (Biesalski et al., 2007). To a lesser degree, BCDOII is also involved in the conversion of β-carotene to utilizable retinal content in the eye.

Once inside the RPE, all-*trans* retinol attaches to CRBP. Conversion of CRBP-all-*trans*-retinol to stable all-*trans*-REs (mainly retinyl palmitate) is performed by LRAT (Gropper et al., 2009). Understanding of the importance of LRAT was obtained from a study of mutant mice with the LRAT gene ablated. The LRAT deficient mice had RE stores in adipose tissue but lacked stores in liver, eyes, lungs, testes, skin, or spleen (Batten et al., 2004; Liu and Gudas, 2005; O'Byrne et al., 2005; Liu et al., 2008). Arguably the most critical regeneration step in the visual cycle, isomerization of all-*trans*-retinyl ester to 11-*cis*-retinol occurs through the action of the isomerohydrolase RPE65 (Redmond et al., 2001; Moiseyev et al., 2006). A genetic mutation in RPE65 has been shown to lead to various forms of retinal dystrophies in previous studies, indicating its crucial nature in the visual cycle (Marlhens et al., 1997; Morimura et al., 1998; Thompson et al., 2000; Thompson and Gal, 2003).

11-*cis*-retinol undergoes a series of dehydrogenation steps through the action of retinol dehydrogenases (RDH5, RDH10, RDH11) to form 11-*cis*-retinal completing the catalytic portion of the retinoid cycle (von Lintig et al., 2010). Cellular retinaldehyde-binding protein (CRALBP) protects 11-*cis*-retinal as it is transported to the apical surface of the RPE plasma membrane and subsequently transposed into the interphotoreceptor matrix onto an inter-photoreceptor retinol binding protein (IRBP), a soluble lipoylcoprotein containing three or four retinoid binding sites per molecule (von Lintig et al., 2010; McClinton, 2012) (Fig. 1-6). IRBP and 11-*cis*-retinal move to the outer segment of the photoreceptor where 11-*cis*-retinal binds via a Schiff-base linkage to a lysine amino acid residue of opsin, forming the imperative chromophore, rhodopsin (Gropper et al., 2009).
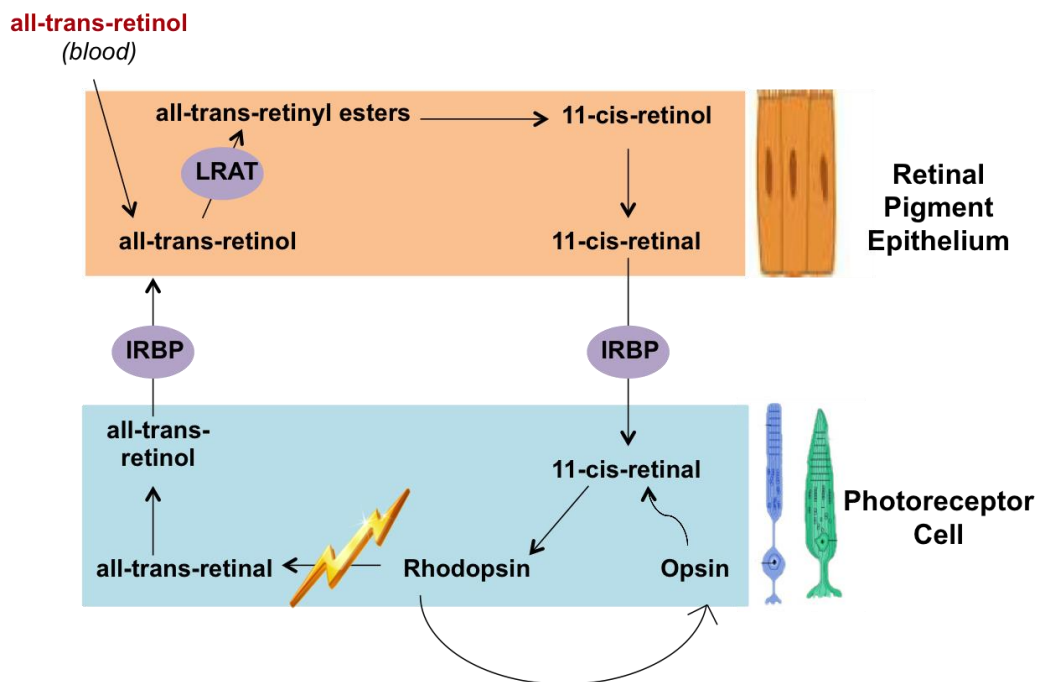
Figure 1-6: Conversion of vitamin A in the visual cycle (Image modified from McClinton, 2012). IRBP, interphotoreceptor retinoid binding protein; LRAT, lecithin : retinol acyltransferase.

When light enters the retina, the electromagnetic radiation activates and cleaves rhodopsin into opsin and all-*trans*-retinal, triggering a cascade of reactions, all occurring within picoseconds via the involvement of the G-protein secondary messenger family (Lien and Hammond, 2011). This is the only light-dependent step in the visual cycle. In order for the visual recycling process to continuously occur and regenerate 11-*cis*-retinal, all-*trans*-retinal must be reduced to all-*trans*-retinol by an NADPH-dependent retinol dehydrogenase (Gropper et al., 2009). Transport of all-*trans*-retinol across the interphotoreceptor matrix back into the RPE requires IRBP and CRBP. Once inside the RPE, two fates of all-*trans*-retinol exist; all-*trans*-retinol may be converted into all-*trans*-REs and further 11-*cis*-retinal, in preparation to complete the visual cycle again or, all-*trans*-retinol can subsequently be stored.

Cone visual cycle

Although within the human retina, the presence of rod cells is more than 30 times greater than that of cone cells, cone cells play a crucial role in higher light intensity vision, visual acuity, and color vision (Mustafi et al., 2009). Therefore, cone cells likely have a different regenerative visual cycle route than rod cells due to the variation in light response kinetics between the two. Mata et al. (2002, 2005) hypothesize the involvement of Müller cells, the main glial cells, in the recycling of visual chromophores in cone photoreceptors. Supportive of this hypothesis, following treatment of mice retinas with L-α-aminoadipic acid, a potent gliotoxin, rod function was unaffected but cone cell recovery was hindered (Wang & Kefalov, 2009). The proposed cone cycle (Mata et al., 2002; Muniz et al., 2007), highlighted in the upcoming paragraph, remains speculative and requires further molecular research to substantiate the evidence. Similar to the rod visual cycle, photoisomerization of 11-*cis*-retinal bound with cone opsins to all-*trans*-retinal, initiates the pathway. Reduction of all-*trans*-retinal is catalyzed by cone retinol dehydrogenase. Müller cells uptake all-*trans*-retinol and isomerize it to 11-*cis*-retinol via

an RPE65-independent or dependent mechanism (yet to be confirmed); ARAT then esterifies it to 11-*cis*-REs or it may be bound to apo-CRALBP (Saari, 2012). Mobilization of 11-*cis*-RE stores is completed by REHs, which are activated by apo-CRALBP to yield 11-*cis*-retinol that is taken up by cone inner segments (Mata et al., 2002). Oxidation of 11-*cis*-retinol ensues, producing 11-*cis*-retinal, and subsequent visual pigments (Saari, 2012). The main proposed difference between the rod and cone visual cycle is the ability of cone cells to oxidize 11-*cis*-retinol for pigment regeneration, whereas rod cells rely solely on 11-*cis*-retinal (Saari, 2012). Thus, the product of the cone cell cycle is only capable for cone cell regeneration, eliminating competition between the two cycles and providing ample and efficient regeneration to cone cells. As previously mentioned, further research on this topic is critical to solidify these hypotheses presented.

Other carotenoids in visual function

Unlike other carotenoids, lutein and zeaxanthin exist in high concentrations in the macular pigment layer of the retina (Widomska and Subczynski, 2014). Specifically, xanthophylls accumulate within photoreceptor axons (Snodderly et al., 1984) and photoreceptor outer segments (Rapp et al., 2000; Sommerburg et al., 1999). Xanthophylls within photoreceptor outer segments comprise 10-25% of total xanthophylls within the entire retina (Rapp et al., 2000; Sommerburg et al., 1999), although rod outer segments contain xanthophylls at a concentration greater than 70% in other retina layers (Sommerburg et al., 1999). Research has also suggested xanthophylls are present within Müller cells (Gass, 1999). Due to their substantial nature in the ocular structure, numerous studies have evaluated the beneficial role of high lutein and zeaxanthin dietary intake and serum concentrations in reducing the risk of age-related macular degeneration (AMD) and cataracts (Carpentier et al., 2009; Age-Related Eye Disease Study Group, 2001; Krinsky et al., 2003). Due to the antioxidant nature and ability to absorb blue light

and ROS by lutein and zeaxanthin, it is of interest to pursue further research to elicit their function in the retina fully.

Existing research illustrates both the functional and protective aspects of lutein and zeaxanthin within the retina layers to combat ROS and phototoxic short-wavelength blue light by acting as a filter and an antioxidant. A study conducted by Ma and others (2009) provided human participants with either 6 or 12 mg of lutein per day for 12 weeks and assessed their plasma carotenoid levels and visual performance indices. Their results indicate a correlation between increased plasma lutein with improved glare sensitivity. Richer et al. (2011) supplemented participants with preexisting conditions of AMD with 8 mg zeaxanthin, 8 mg zeaxanthin + 9 mg lutein, or 9 mg lutein alone per day for 1 year. They measured the macular pigment optical density (MPOD) levels within each group at baseline, 4, 8, and 12 months. All treatment groups increased MPOD from baseline, however, the lutein supplemented group showed the greatest increase in MPOD. In addition, zeaxanthin supplementation also improved participant's self-assessed driving performance, visual acuity and foveal shape discrimination (Richer et al., 2011).

A number of other factors exist within the retina, besides being exposed to a constant light source, that positively influence the nature of the retina in acting as a pro-oxidative environment susceptible to oxidative stress: 1) The retina is highly vascularized resulting in high oxygen tension between the photoreceptors; 2) Light stress may be more prevalent at the fovea due to increased cellular density and greater metabolism; 3) Rods and cones are rich in long-chain polyunsatured fatty acids, particularly DHA, which are highly susceptible to oxidation; and 4) Light induced reactions of specific compounds stimulate the generation of free-radicals, highly reactive and damaging species (Lien and Hammond, 2011).

It is becoming of greater interest to conduct clinical trials to assess the beneficial effects of lutein and zeaxanthin supplementation on retinal function. The current studies show an increase in visual acuity and an increase in MPOD (Stringham and Hammond, 2005; Richer et al., 2011; Sabour-Pickett et al., 2014; Wenzel et al., 2006; Vishwanathan et al., 2009). However, although it is known the important role of carotenoids in the visual cycle and as antioxidants in the isolated form, no known human studies to date have uniquely assessed the impact of carotenoid supplementation as a whole food in humans with and without eye conditions.

Carotenoids in chronic disease

Many chronic diseases, such as cardiovascular disease (CVD) and cancer, stem from the actions of ROS creating oxidative stress. One of the ways to combat oxidative damage is through the action of antioxidants, such as carotenoid compounds. Carotenoids prove to be one of the most efficient singlet oxygen quenchers and ROS scavengers in cellular lipid bilayers (Fiedor and Burda, 2014).

Epidemiological findings suggest a positive correlation between a higher fruit and vegetable intake, rich in carotenoids, and a reduction of morbidity and mortality related to CVD, however intervention studies are less consistent (Fiedor and Burda, 2014). Randomized clinical trials evaluating the effect of β-carotene supplementation on CVD show mixed results, finding an insignificant difference in CVD incidence (ATBC group, 1994), an increase in risk of post-trial myocardial infarction (Törnwall et al., 2004), and increased risk of mortality in heavy smokers (Liu et al., 2009). Results of the studies may vary due to supplement doses, study duration, genetics and health conditions. Conversely, a pro-oxidant mechanism of β-carotene has shown to increase the risk of lung cancer in chronic smokers and alcoholics due to the strong interference of carotenoids with unhealthy lifestyle choices (Liu et al., 2009; Fiedor and Burda, 2014).

Carotenoid supplementation studies

Much of the metabolic and bioavailability data presented here is based on healthy animal supplementation trials, however, it is of interest to examine whether present human studies elicit similar results following carotenoid supplementation to ensure carotenoids are available for metabolism to produce beneficial results.

In recent studies, carotenoid supplementation was shown to significantly increase levels of their metabolites in the serum (Rotenstreich et al., 2013) and in the eye (Bernstein et al., 2001). In earlier studies, quantification of carotenoids in the human eye was unable to be detected beyond trace amounts even though there were significant quantities in the plasma. Bernstein et al. (2001) were able to quantify numerous dietary metabolites within ocular tissues including, 3R,3′S,6′R-lutein-3′-epilutein, ε-carotene-3,3′-diol, 3-hydroxy-β-ionone, 3-hydroxy-14′-apocarotenal, ε,ε-carotene-3,3′-dione, 3-hydroxy-β,ε-caroten-3′-one, and 3′-hydroxy-ε,ε-caroten-3-one, reiterating the critical role of carotenoids in visual function and protection against light-induced oxidative damage (Bernstein et al., 2001). Many other cleavage products are believed to exist, but have yet to be identified, possibly due to lack of appropriate methods.

Previous research exists illustrating the role of isolated β-carotene supplementation on visual function in individuals with retinitis pigmentosa (Rotenstreich et al., 2013). ERG b-wave responses increased 40% from baseline in those receiving β-carotene supplementation in comparison to a 16% decrease in the control group. Similarly, the importance of vitamin A can be illustrated in a study conducted by McBain et al. (2007). Following a state of vitamin A deficiency, improved ERG responses can be seen in as little as 3 days following administration and almost normalized after 12 days. This evidence illustrates the crucial nature of vitamin A and carotenoids in visual function and reinforces the potential benefit of synergistic carotenoids in retina health.

Computer Vision Syndrome

Although the presence of the computer within the work place has accomplished many beneficial advances, it is estimated that in the year 2000 more than 75% of all jobs involved computer usage, indicating an increased amount of ocular problems and discomfort (Blehm et al, 2005). Additionally, personal use of computers, tablets, smart phones, laptops, and other electronic devices has also increased the time spent viewing a computer screen. Viewing text on the computer screen is more visually demanding than viewing text on paper due to the resolution, contrast, glare, working angles, distances, and image refresh rates of the screen, creating frequent saccadic eye movements, accommodation and vergence of the visual system (Verma, 2001). The range of symptoms associated with computer use and visual discomfort has been coined as CVS. The AOA (2014) defines CVS as "the complex of eye and vision problems related to near work, which are experienced during or related to computer use."

Symptoms

Common CVS symptoms can be divided into three main categories (Blehm et al., 2005; Sheedy, 1996; Sheedy, 2000): (1) eye-related symptoms (e.g. dry eyes, watery eyes, irritated or burning eyes); (2) vision-related symptoms (e.g. eyestrain, eye fatigue, headache, blurred vision, double vision); and (3) posture-related symptoms (e.g. neck pain, shoulder pain, back pain). The five most common reported CVS symptoms are eyestrain, headache, blurred vision, dry eyes and neck/back pain (Yan et al., 2008).

There are many variable factors associated with video display terminal (VDT) use that can provoke these symptoms. Environmental factors such as dry air, ventilation fans, dust, and static buildup within the office setting can increase the dryness and chemical imbalances on the cornea (Blehm et al., 2005). Sex and age influence the prevalence of dry eye due to a decreased tear production with age, along with a higher incidence of dry

eyes amongst females (Blehm et al., 2005). Additionally, bright lighting and glare adversely affect the computer user's ocular discomfort (Blehm et al., 2005).

CVS is a largely vague and unknown syndrome, but is one that is rapidly growing. CVS symptoms are likely a result of the combination of ocular, extra-ocular, and accommodative mechanisms. Ocular surface mechanisms produce dry eyes, redness and a burning sensation. The pathophysiology behind this mechanism is multifactorial; including corneal dryness due to both environmental and physiological factors, reduced blink rate, incomplete blinking, increased surface area exposure on the cornea, and decreased tear production due to age, contact lenses, medications and various diseases (Loh and Reddy, 2008). Extra-ocular symptoms are related to improper ergonomic placement of the computer and posture that cause musculoskeletal symptoms such as neck/back pain and headaches. Accommodative mechanisms of CVS create symptoms such as blurred vision and double vision. These symptoms can arise from pre-existing visual processing problems such as hyperopia, astigmatism or myopia (Yan et al., 2008).

Prevalence

The computer user population in 2003 in the United States was estimated at 188 million people, with a total national population of 275 million people (Yan et al., 2008). Therefore, approximately 68% of Americans use the computer as part of their daily life. This corresponds to the estimates produced by the AOA (2014), with approximately 70-75% of computer users experiencing CVS symptoms. Various studies have attempted to estimate the number of computer users suffering from CVS. The safest estimate of the CVS population in the United States is at least 15-47 million with a minimum of 14-23% of users experiencing varying degrees and types of CVS symptoms (Yan et al., 2008), while a review conducted by Thomson (1998) indicated as many as 90% of computer users experienced some sort of visual discomfort. Numerous individuals mistreat CVS

symptoms with medications such as Advil, while many optometrists and affected users are unaware of the syndrome in general. The AOA estimates that 12 million eye examination visits each year are due to computer related problems (2014). This corresponds to 1 of every 5 patients who seek eye examinations. These statistics indicate the pressing need to identify a therapeutic remedy that will provide patients with reprieve and potentially attenuate the development of CVS all together.

Extensive research with insight into populations at higher risk for developing CVS has not been conducted, however, two populations have been identified to be at greater risk; users with pre-existing vision problems and children. Computer users with pre-existing vision problems (accommodative disorders, binocular vision disorders and refractive error) develop CVS more quickly and symptoms of greater severity than those without (Yan et al., 2008). Another population at an increased risk for development of CVS is children. Children spend time on the computer not only at home but also at school. Hoenig (2002) estimates that of the 37 million children using the computer, the average American child will spend 1-3 hours per day on the computer. The author found a correlation between children who use the computer for extended periods of time and premature myopia. There are a multitude of unique reasons behind the susceptibility of children in developing CVS. Children often do not have the required self-control to limit the amount of time they spend on the computer nor introduce regularly scheduled breaks. This prolonged activity increases their susceptibility to develop CVS. Children also lack knowledge and experience often resulting in misinterpretation of visual problems from the computer screen as normal. Therefore, the blurred vision, astigmatism or myopia/hyperopia in due part from the computer, may be assumed to be normal by the child (Yan et al., 2008). Additionally, computer workstations are generally set up to accommodate the adult user, hence, incorrectly set up for children (Hedge, 2005).

Overall, computer viewing requires interaction of numerous visual skills that children have not fully developed at the present life stage (Anshel, 2000b).

Literature examining the relationship between the types of computer users and the vulnerability of such users in developing CVS is quite limited. It is of interest to know whether factors such as age, gender, occupation, and socioeconomic status are influential in determining who is at a greater risk for developing CVS.

Treatment

Workplace health authorities have suggested a variety of multidirectional ergo-ophthalmologic approaches in an attempt to mitigate the ocular discomforts experienced as a result of VDT use. Successful treatment involves ocular therapies in addition to positive computer workplace modification. As previously mentioned, various lighting can negatively affect computer users. One solution to this problem is to ensure equalized brightness throughout the visual field of the user (Abelson and Ousler, 1999). The type of lighting has also been shown to be a factor in creating reflection and glare off the monitor. Intense fluorescent lights and bright streaming windows should be dimmed for the highest functional capacity of the eyes (Blehm et al., 2005). Anti-glare filters are an external modification that is available to computer users although studies indicate mixed results in their efficacy (Blehm et al., 2005). Screen filters may be able to provide users with relief from visual discomforts but do not appear to reduce the occurrence of asthenopia (eye strain) (Scullica et al., 1995).

Computer users often contort their body to an uncomfortable position in order to gain a better view of the computer screen leading to poor posture, which can account for muscular and ocular stress following extended period of computer use. Initially, the recommended viewing distance between the eye and the screen was between 16 to 30 inches (Shahnavaz, 1984; Von Stroh, 1993). Recent studies highlight the efficacy of

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further viewing distances, between 35-40 inches, in reducing visual discomforts (Blehm et al., 2005). Angle of the computer screen is also a crucial factor in preventing muscular strain of the upper trapezius and neck muscles (Blehm et al., 2005). The computer screen should be 10-20 degrees below eye level (5-6 inches) (Loh and Reddy, 2008).

Long extended periods of computer use without breaks are a significant factor in ocular discomfort. Traditionally, office workers have two 15 min breaks throughout the day. The National Institute of Occupational Safety and Health has shown a decrease in discomfort and increase in productivity with more frequent work breaks (Sellers, 1995). Anshel (2005) suggests following the 20/20/20 rule; after working on the computer for 20 mins, the user should look 20 feet away for at least 20 sec. These frequent breaks allow for the visual accommodative system to relax and restore, preventing the occurrence of eyestrain, however, its effectiveness is not known.

Dry eye is a significant factor contributing to the discomfort of computer users. Dry eyes can be in part due to environmental surroundings (arid, dust), decreased tear production as a result of increased exposed corneal surface area, contact lens use, and decreased blink rate. Tsubota and Nakamori (1993) studied the blink rate of participants in a relaxed state and while using the computer. Their data showed the mean ($\pm$ SD) rate of blinking was 22 ± 9 per min in relaxed conditions and 7 ± 7 per min whilst viewing the computer terminal. This is almost a 33% reduction in mean blink rate per min. To combat dry eyes, lubricating drops are often used to rewet the corneal surface, increase tear volume, and maintain proper environmental conditions on the ocular surface (Blehm et al., 2005). Although over-the-counter lubricating drops can provide temporary relief to the user, a high viscosity drop can actually reduce visual acuity (Blehm et al., 2005). However, despite the high rate of over-the-counter relief medications, the majority of the patients were left unsatisfied with the therapeutic results obtained (Shimmura et al.,

1999). In a recent clinical trail on dry eye syndrome, supplementation of sea buckthorn oil positively affected dry eye by attenuating tear film osmolarity and alleviating dry eye symptoms (Larmo et al., 2010). This indicates that some CVS related symptoms could be treated with nutrient supplementation.

Computer eyeglasses may be another potentially successful treatment option for certain CVS symptoms. Computer eyeglasses differ from regular eyeglasses as they are specifically designed for the computer workplace and have a special lens design, power, and tint/coating (Yan et al., 2008). Progressive lenses still create annoyance for the user while searching for the "sweet-spot" on the lens. Occupational progressive lenses have a larger area on the top portion of the lens for mid distance viewing (computer screen) and a bottom half for near viewing (keyboard) (Wimalasundera, 2006).

Each year CVS diagnoses and treatments translates to 2 billion dollars in costs in the United States (Abelson and Ouster, 1999). This is a costly, widely growing epidemic with no absolute therapeutic treatment or prevention method. Although numerous short-term external modifications to the VDT workplace have been shown to provide reprieve to the user, no known studies have examined whether dietary intake can restore visual function or mitigate problematic ocular symptoms in the chronic computer user. Due to the high costs and decreased productivity associated with the symptoms of CVS, it is cost beneficial and therapeutically beneficial to identify a possible nutrition intervention to prevent and mitigate CVS. It is of interest to study if diet can mitigate the symptoms of CVS and potentially develop the basis of a preventative dietary recommendation for long-hour computer users.

Carrot as a source of vitamin A and carotenoids

Carrots are one of the most widely consumed vegetables, providing 14% of the total vitamin A consumption and a rich source of carotenoids including β-carotene, α-

carotene, lutein, and zeaxanthin (Desobry et al., 1998). It is known that the carrot provides many nutritious and beneficial compounds important for the eyes, however, no interventional studies have examined the effects of the whole carrot on visual function. Carrots are also a source of dietary fiber (2.9g/100g of raw carrot) (Table 1-1), a nutrient that exhibits total and LDL cholesterol lowering properties, balances intestinal pH and regulates intestinal glucose absorption and blood glucose levels.

Table 1-1: Raw carrot nutrient information (100g)

Nutrient name	Per 100 g
Protein (g)	0.93
Total Fat (g)	0.24
Carbohydrate (g)	9.58
Energy (kcal)	41
Fiber, total dietary (g)	2.4
β-carotene (μg)	8285
α-carotene (μg)	3477
Retinol (μg)	0
Retinol activity equivalents, RAE	83
Lutein and zeaxanthin (μg)	256
Lycopene (μg)	1
β-cryptoxanthin (μg)	0

Information modified from Health Canada: Canadian Nutrient File; Food Code 2380 (Health Canada. 2010).

Heinomen (1990) conducted a study on 19 carrot cultivars and observed that a β-carotene level between 1200-2300 μg per 100g of carrot is adequate to meet the human daily vitamin A requirement. Since carotene is a highly oxidizable and unstable compound, Desobry et al. (1998) found that the best preservation of β-carotene is via a freeze-drying and encapsulation method. Simon and Wolff (1987) demonstrated that the

amount of α-carotene may vary from 15 to 40% of total carotenoids and β-carotene may be from 44 to 79%. Table 1-1 demonstrates the nutrition values of the raw carrot composed by Health Canada: Canadian Nutrient Files (2010).

Carrot as a health food

Vegetables and fruit are key foods to be included into the human diet as they provide specific nutrients, dietary fibers, and phytochemicals. The greatest nutritional research has been focused on the phytochemical properties of carrots, however, carrots are also a great source of fiber. In perspective, 100g of raw carrots, provides a female aged 19 to 30, with 11% of their total daily fiber (Arscott and Tanumihardjo, 2010). Extensive research has been conducted examining the inverse relationship between dietary fiber intake and the risk of CVD. Carrots are a great source of soluble fiber, a type of fiber hypothesized to have hypocholesterolemic properties. Additionally, fiber has the ability to delay gastric emptying, reduce postprandial responses and increase satiety, all factors which improve glucose metabolism, a key component in reducing blood pressure and lowering the risk for developing diabetes (Satija and Hu, 2012). Therapeutic animal models have elicited promising results with regard to carrot intake and its health benefits in terms of CVD, cancer, glucose metabolism, and obesity. A study conducted by Nicolle and others (2003) investigated the effect of 3-week carrot supplementation on lipid metabolism and antioxidant status in a rat model. Results from the study illustrate a 44% decrease in liver cholesterol in conjunction with a 40% decrease in liver triglycerides (Nicolle et al., 2003). Carrot supplementation also decreased excretion of ROS indicating an increase in antioxidant status (Nicolle et al., 2003). Clinical trials examining the same responses with regard to carrot consumption are sparse indicating the need for further research (Arscott and Tanumihardjo, 2010).

Many studies have illustrated the effects of purified vitamin A, β-carotene, fiber, lutein and zeaxanthin supplementation, all compounds found within the carrot; however, the remedial effect of the whole carrot, which contains numerous carotenoids and other nutrients, has not been tested. Additionally, the impact of the whole carrot with respect to eye health has not been tested. Therefore, it is of interest to know whether carrot carotenoids possess a beneficial role involved in rod and cone cell function; and if β-carotene and lutein present in the carrot will improve or alter the bioavailability of vitamin A within the serum to potentially mitigate ocular problems associated with CVS.

Chapter II. RESEARCH PLAN

Rationale

CVS, an ocular related group of discomforts characterized by the prolonged use of the computer, is a widely growing epidemic in society with no sight of slowing down. With the increased prevalence of computers in society, be it through laptops, smart-phones or tablets, there is no escaping computer use. In Canada in 2001, 8.3 million people used the computer at work and 78% of those users did so daily (Statistics Canada, 2010). Of these computer users, it is estimated that 70-75% experience some type of ocular discomforts related to computer use (AOA, 2014). This is likely an underestimate due to the fact that many computer users often try to self-remediate their symptoms through medications, such as Tylenol, Advil or eye drops. The most common reported symptoms are dry eyes, blurred vision, headaches, and eyestrain, as outlined by the Ocular Surface Disease Index (OSDI) questionnaire, which may potentially hinder users quality of life and productivity. Due to the relatively new nature of CVS, it is unknown whether long-hour computer users exhibit any retinal problems, which may possibly be promoting users symptoms. Numerous environmental and ophthalmological modifications exist that can provide the user with short-term reprieve; however, users were often not completely relieved of their symptoms. These symptoms remain a major challenge, indicating the need for therapeutic strategies, such as nutrition.

It is known that vitamin A plays an integral role in the visual cycle as a component of photoreceptor retinal chromophores, required for photoisomerization. Since the body cannot produce these compounds, a constant supply of vitamin A is needed from the diet. Vitamin A deficiency is the leading cause of xerophthalmia worldwide, however, upon vitamin A supplementation, retinal function has been shown to normalize, illustrating its essential role in visual function (McBain et al., 2007; da

Rocha Lima et al., 2014; Christian et al., 1998). These studies indicate vitamin A supplementation can be used to improve visual function in normal and deficient models, however, whether these nutrients also help individuals with CVS ocular symptoms has not been studied.

The carrot has been a well-known health food for the eyes for centuries. It is a leading dietary vegetable around the world and one of the richest sources of carotenoids (β-carotene, α-carotene, lutein, and zeaxanthin). These carotenoids present in the carrot act as antioxidants and a dietary source of vitamin A. Extensive research studying the effects of isolated carotenoids present in the carrot, such as xanthophylls and carotenes, on visual function has been examined showing promising results. Studies have also shown improvements to MPOD and visual function in both diseased and healthy models following lutein and zeaxanthin supplementation (Richer et al., 2011; Sabour-Pickett et al., 2014; Wenzel et al., 2006; Vishwanathan et al., 2009). Similarly, β-carotene supplementation improved ERG b-wave responses by 40% from baseline in individuals with retinitis pigmentosa (Rotenstreich et al., 2013). Interestingly, the whole food matrix of the carrot has been rarely studied in conjunction to visual function. A very recent murine model conducted in our laboratory showed significant improvements in visual function in healthy rats following carrot powder supplementation in the diet (McClinton et al., 2014). Since visual discomforts are one of the main complaints from computer users, it is of interest to see whether similar beneficial retinal improvements via carrot powder are seen in chronic computer users. Additionally, there has been no information about the retinal function of long-hour computer users, which can provide insight into the physiological aspects of CVS. This study will examine the effects of the carrot (a whole food) as a prevention or alleviation therapy for CVS, and identify retina health of individuals with CVS.

Hypothesis

The working hypothesis of this research is that consumption of carrot powder in a food will improve retina function in CVS by increasing plasma carotenoids and vitamin A levels. More specifically,

Whole carrot powder supplementation will:

1. Improve retinal function in CVS individuals i.e.) increase response to light stimulation.
2. Increase retinoid and carotenoid levels in the serum.
3. Relieve symptoms of CVS.

Objectives

The objectives of this investigation are to determine whether carrot powder consumption improves retina function in CVS.

This study will test the hypothesis with four primary objectives:

1. To examine if carrot carotenoids influence the inner and outer retina function.
2. To measure diet induced changes in carotenoids, retinoids, and other metabolites.
3. To examine the association between carotenoids and vitamin A levels in the plasma in CVS.
4. To determine if carrot supplementation can relieve CVS symptoms.

Chapter III. EXPERIMENTAL DESIGN AND METHODS

Experimental Design

Study Design

All study protocols were approved by the University of Manitoba Office of Research Ethics & Compliance Joint-Faculty Research Ethics Board. Recruitment fliers and website ads were posted around the University of Manitoba Fort Garry campus. Study participants were recruited into a double-blind, placebo-controlled, randomized parallel trial using a repeated measure design with PRE (0 weeks), DURING (2 weeks), and POST (4 weeks) assessments to observe the effects of carrot supplementation on visual function and blood and urine parameters. The study duration was chosen based on previous conducted carotenoid and vitamin A supplementation studies, which illustrate that a 4-week supplementation period is substantial to induce changes in plasma concentrations (Meagher et al., 2013; Watzl et al., 2003; Iwamoto et al., 2012; Steck-Scott et al., 2004; Tanumihardjo et al., 2004). Blood and urine samples, and vision related symptoms by a validated questionnaire (Ocular Surface Disease Index) (Ozcura et al., 2007), were collected at PRE, DURING and POST. A full-field ERG measurement for retina function was obtained at PRE and POST. Although food records were not assessed, as the study targeted free-living individuals with no diet changes, participants were asked to refrain from use of fish oil supplements, consuming copious amounts of fish (1+ serving/day), intake of other supplements and limited intake of vitamin A rich foods during the course of the trial.

Potential participants (n=53) were screened for inclusion and exclusion criteria. Inclusion criteria included: i) Participants between 20-65 years of age (working age group) that are otherwise healthy; ii) Use of a computer (desktop, laptop or both) for a minimum of four hours each day for at least four days per week (AOA, 2014); iii) Computer users complaining of a minimum of three symptoms from eye strain, dry eyes,

blurred vision, redness, burning eyes, excessive tears, double vision, headache, glare sensitivity and eye fatigue; iv) Minimum one year exposure to any type of computer use. Exclusion criteria includes: i) Consultation of a specialist for their visual and ocular diseases; ii) Had eye clinical conditions such as Sjogren's syndrome or kerato-conjunctivitis sicca; iii) Used medication associated with dry eye syndrome (e.g. Anti-histamines etc); iv) Use of medication associated with any other chronic diseases; v) Frequent consumption of β-carotene enriched food (two servings per week) e.g. kale, carrots, turnip greens; vi) Currently taking vitamin and/or antioxidant supplements such as vitamin E, vitamin C, β-carotene, lutein, multivitamin tablet.

Participants

No power calculations were conducted, as this was a pilot study, however we were aiming for a total of 30 participants as a study conducted by Julious (2005) provided evidence supporting a minimum sample size of 24 participants in a pilot study is substantial to obtain robust and reliable results. A total of 53 participants were screened prior to commencement of the study, however only 28 met our strict inclusion and exclusion criteria. Of the 28 eligible participants only 20 chose to participate due to time conflicts and personal reasons. Participants were then randomized, by issuing computer-generated numbers, to either the control or carrot group. 9 (90%) participants in the control group and 10 (100%) participants in the carrot group completed the entire protocol. Only one participant did not complete the study as a result of personal reasons. There were no adverse effects, as reported by participants.

The most common reason of ineligibility was due to consumption of vitamin supplement(s) (56%), carrot consumption (48%), and on medications for dry eyes (20%) (Fig. 3-1).
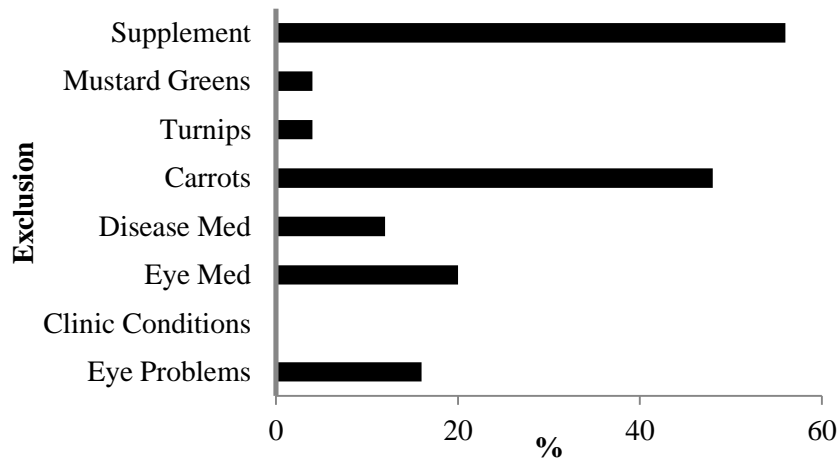
Figure 3-1: Reason screened participants were deemed ineligible. Data expressed as percentage reported (n=25) by all ineligible participants.

Diet Supplements

The participants were randomly assigned to receive either placebo (15g cream of wheat powder) or carrot enriched (15g carrot powder) supplement in the form of an isocaloric yogurt and isocaloric pudding (Table 3-1) every day (7.5g supplement in each) for 4 weeks. All participants received fresh supplement supplies every two weeks to minimize nutrient loss and decomposition, and to prevent spoilage of the dairy supplements. The content of the carrot powder carotenoids in the supplement was measured at 0, 7, and 14 days after the shipment was received and stored in -80°C.

Since the study participants are healthy, 15g of carrot powder was chosen, providing approximately only 33% of the vitamin A RDA, as research has identified over consumption of carotenoids can act as pro-oxidants in the retina by increasing lipofuscin (Hunter et al., 2012; Nowak, 2013). This minimal amount of carrot powder can also be easily obtained through habitual diet consumption.

Based on carotenoid quantification using reverse phase ultra- performance liquid chromatography (UPLC) with photodiode array (PDA) detection there is 41.26mg β-

carotene/100g powder (McClinton, 2012), which provides 6.19mg/15g powder. Since the amount of β-carotene required by the body to produce 1μg RAE of vitamin A is 12μg, and the conversion rate of provitamin A from food form is half that of preformed vitamin A, the supplement provided approximately 257.9 RAE of vitamin A. This represents 28.7% and 36.8% of the vitamin A RDA for an adult male and female, respectively.

Freeze dried carrot powder for the diet was provided by the Food Product Development Centre Portage la Prairie, Manitoba. The yogurt and pudding were produced at the Canadian Food Inspection Agency (CFIA) certified dairy lab at the University of Manitoba. The nutrient content of the powder was measured by SGS Canada Inc. (Vancouver, BC, Canada) and contained protein (9.4%), carbohydrate (68.4%), fat (1.0%), moisture (11.3%), and ash (9.9%). Mineral and fiber content were found to be approximately 3.3g/100g and 23g/100g respectively. This information obtained illustrates similar values, within a range, to the existing data put forth by Health Canada (2010) (Table 1-1).

Table 3-1: Nutritional composition of supplements (/100g)

Nutrient	Control		Carrot	
	Yogurt	**Pudding**	**Yogurt**	**Pudding**
Energy (cal)	74	131	74	131
Total Fat (g)	2	2	2	2
Saturated Fat (g)	1	1	1	1
Protein (g)	4	4	4	4
Carbohydrate (g)	10	25	10	24
Fiber (g)	0	0	2	2
Total Sugars (g)	4	16	7	19
Sodium (mg)	55	55	55	55

Questionnaires

Lifestyle

Measured height and weight were used to calculate body mass index (BMI), and the following classifications were used: obese ($\geq$30.0), overweight (25.0-29.9), normal

weight (18.5-24.9), and underweight (<18.5). Self-reported health statuses were obtained from the questionnaire. Health status was classified as poor, fair, good, very good and excellent.

Frequency and length of computer use of participants were collected to assess computer use in correlation with CVS symptoms. Frequency of computer use was classified as <4 days/wk, 5, 6, and 7 days/wk. Length of computer use was classified as <4 hours/day, 4-6, 6-7, and 7+ hours/day.

Ocular Surface Disease Index Questionnaire

A modified questionnaire was devised using the Ocular Surface Disease Index questionnaire (Ozcura et al., 2007; Schiffman et al., 2000) to assess the self-perceived visual function and discomfort of participants at PRE, DURING and POST.

Experimental Methods

Blood Parameters

Fasted blood was collected via venipuncture located in the antecubital fossa, and centrifuged (1932 × g, 10 min, at 4°C). Plasma was aliquoted into 1.5 and 2.0mL eppendorf tubes and stored at -80°C until analysis. Urine (10mL) was also collected at the same time and stored at -80°C for further analysis.

Plasma Glucose

Plasma glucose was measured using an auto-analyzer, Vitros® 350 Chemistry System (Ortho Clinical Diagnostics).

Plasma Lipids

Plasma lipid profiles (triglyceride, HDL, LDL, total cholesterol (TC) and very low density lipoproteins (VLDL)) were measured using an auto-analyzer, Vitros® 350 Chemistry System (Ortho Clinical Diagnostics).

Electroretinography

Retinal function was determined using full-field ERG with the UTAS-4000 data system (LKC Technologies Inc., Gaithersburg, MD). To ensure maximum sensitivity, participants were dark-adapted for 20 min prior to recording. To assess rod and cone function, mixed scotopic response, photopic response, and photopic flicker response were used on the retina, as previously established in our laboratory (Suh et al., 2009). ERG data was analyzed for both right and left eyes of each participant. Data was reported as baseline response (PRE) and following 4 weeks of treatment (POST) for each eye (right and left) of each participant to show the effects of supplementation. Schematic representation of full-field ERG apparatus is in Figure 1-5.

Participants were prepared for bilateral ERG recording under dim red light. Pupils were dilated using a drop of 1% tropicamide applied onto the corneal surface 20 min prior to recording to allow adequate time for pupil dilation. The forehead surface, right ear lobe and inside and outside corners of the participants' eyes were cleaned using an electrode skin prep pad (70% isopropyl alcohol and pumice) to ensure maximal conductance. DTL plus electrodes were applied to the corneal surface just beneath the bottom eyelid and secured with adhesive pads to the corners of the eye. An electrocardiograph (ECG) electrode was adhered to the forehead and a reference electrode was attached to it. Electrode gel was added on the right ear lobe to connect the ground electrode.

Dark-adapted ERG

Dark-adapted, rod-driven intensity responses were generated by eliciting a series of flash stimuli with increasing intensity (Suh et al., 2009). Stimuli consisted of a single white flash (6500 K, xenon bulb), 10μsec in duration, presented 3 times (to verify response reliability and consistency and obtain an average) at 10 to 14 increasing steps of intensity ranging from -3.7 to 1.89 log sc cd·sec/m^2 (logarithm of scotopic candela sec/meter square) in luminance. Inter-stimulus intervals increased from 10 sec up to 2 min at the greatest intensity in order to allow for maximal rod recovery between flashes. For all ERG recordings, a-wave (representing photoreceptor activity) was measured as the difference between the baseline at 0 sec and the lowest point of the negative trough. B-wave amplitude (representing post-synaptic activation of ON-bipolar cells and Müller cells in the inner retina) was measured as the difference between the a-wave negative trough and the apex of the positive b-wave. The summed amplitude of OPs (representing activity of inner retina neurons initiated by amacrine cells) were calculated using commercial software (EMWin from LKC Technologies Inc., Gaithersburg, MD).

Light-adapted ERG

Following scotopic recordings, participants were exposed to a 30 sc cd·sec/m^2 background light prior to assessing cone-driven intensity responses by presenting single flashes (6500 K, 10 μsec in duration) at 10 increasing intensity steps ranging from -1.22 to 2.86 log sc cd·sec/m^2. A total of 5 flash stimuli responses were averaged for each intensity. Amplitudes were calculated in the same fashion as described for scotopic responses.

Carrot powder carotenoid composition and stability measurement

Chemicals, materials and preparation of standard stock solutions

Carotenoids, all-*trans*-β-carotene and *trans*-β-apo-8'-carotenal, and retinoids, all-*trans*-retinol, and retinyl acetate were purchased from Sigma-Aldrich (Oakville, ON). Lutein, zeaxanthin and α-carotene reference standards were generously donated by DSM Nutritional Products (Basel, Switzerland). All compounds were ≥95.0% (HPLC) grade. LC-MS grade acetonitrile, water, methanol and methyl-t-butyl ether were obtained from Sigma-Aldrich (Oakville, ON). In order to minimize degradation, standards and solutions were prepared on the same day as extractions were. All stock solutions were prepared using HPLC grade chloroform : methanol (1:3, vol/vol) (Sigma-Aldrich, ON, Canada) and stored at -80°C.

Sample preparation

Carrot powder and diet samples

All sample preparations occurred under dim red light to prevent oxidation of the carotenoids. The 4 compounds examined in the carrot powder, all-*trans*-β-carotene, α-carotene, zeaxanthin and lutein, were extracted using the method previously developed in our laboratory (McClinton, 2012). Simply, 0.665g of supplement enriched with carrot powder or cream of wheat powder were extracted in duplicate with 12 mL of chloroform : methanol (2:1, vol/vol), and 15 mL of hexane. The internal standard, *trans*-β-Apo-8'-carotenal (4μg), a known vitamin A metabolite in mammalian tissue but not in plants, was added to each sample to determine the recovery and account for any loss of sample during extraction handling procedures. Each sample mixture was homogenized to release any carotenoids from the food matrix. All sample mixtures were vortexed for 1 min and sonicated for 15 min (Branson 3510, Danbury, CT) and then centrifuged at $1236 \times g$ for

15 min (Sorvall Legend RT, Thermo Scientific, Waltham, MA). The upper organic phase was removed. This extraction procedure was repeated once more. Organic phases were pooled and evaporated to dryness under a nitrogen gas stream. The residue was redissolved in 1 mL of chloroform : methanol (1:3, vol/vol), which was filtered through a 4mm syringe filter with a 0.2μ pore diameter. Ten μL of the injection mixture was injected into the C30 Carotenoid (3μm; 4.6 cm x 250 mm, Waters Ltd, Lachine, QC) reverse-phase column. Carrot powder samples yielded a 68.0% recovery for carotenoids. Triplicates were run and had intra-assay CVs of 37.3%, 24.5%, 17.7%, and 3.1% for α-carotene, β-carotene, lutein, and zeaxanthin respectively.

Plasma samples

The extraction of carotenoids/retinoids from plasma was based on the method previously established in our laboratory (McClinton, 2012). The retinyl acetate internal standard (0.5μg/10μL) and 0.01% butylated hydroxytoulene (BHT) in ethanol, were added to 500μL of plasma. A 2mL mixture of chloroform : methanol (2:1, vol/vol) and 3mL of hexanes was then added to each aliquot, vortexed for 30 sec and centrifuged at $1236 \times g$ for 15 min. The upper organic phase was separated and evaporated to dryness under a nitrogen gas stream and transferred with two rinses of chloroform : methanol (2:1, vol/vol) to subsequent gas chromatography (GC) vial. The extraction volume was filtered through a 4mm syringe filter with a 0.2μ diameter pore. The GC vial was washed twice and transferred to the corresponding GC vial insert and evaporated to dryness. The remaining residue was redissolved in 100μL chloroform : methanol (1:3, vol/vol).

Chromatographic conditions

To quantify carotenoids and retinoids, the chromatography was performed on a Waters Acquity UPLC system coupled with photodiode array detection (UPLC-PDA),

equipped with Acquity console software and MassLynx 4.1 (Waters Corp., Milford, MA). The column oven and autosampler temperatures were maintained at 35ºC and 21°C, respectively. The autosampler door was covered to minimize light exposure. Carotenoids and retinoids were separated using a C30 Carotenoid (3μm; 4.6 cm x 250 mm, Waters Ltd, Lachine, QC) reverse-phase column, with a gradient mobile phase at 0.3 mL/min flow rate consisting of methanol : methyl t-butyl ether : HPLC grade water (81:15:4, by vol, solvent A) and methanol : methyl t-butyl ether : HPLC grade water (6:90:4, by vol, solvent B).

Carotenoid and retinoid run conditions

Run conditions for carotenoid and retinoids were as follows: Initial, 100% A, held until 15 min; gradient to 30% A, 70% B from 15 min to 24.5 min; re-equilibration to initial composition of 100% A from 24.5 min to 25 min with a hold at 100% A for 5 mins. The total run time was 30 min. Detection wavelength was set at 320-460 nm for all standards and carotenoid and retinoid compounds.

Identification of retinoids and carotenoids was determined by comparing retention times and visible spectra with corresponding standards. To quantify the carotenoids and retinoids, calibration curves with known amounts of the standards in duplicate injections were used. The reference peak area was used to plot the quantity of each standard compound over the area of the internal standard. Using the linear regression equation, concentrations found within the serum and diet were calculated through the use of the ratio of the known compound and the internal standard, retinyl acetate. A few examples of calibration curves are shown in Figure A-1. Linear regression values for α-carotene, β-carotene, lutein, zeaxanthin, all-*trans*-retinol and all-*trans*-retinal were >0.96. Table A-1 includes a list of all quantification equations and associated regression values used for analysis. To ensure intra- and inter-day reproducibility, triplicates of samples were run at

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various time points throughout the day via UPLC. Intra-day stability was assessed from measurements obtained within the same day, compared to inter-day stability, which was assessed from measurements obtained on five different days during UPLC analysis. Detection limit for α- and β-carotene was 25ng/injection with a signal to noise ratio (S/N) of 3.6 and 6.0, respectively. Detection limit for lutein and zeaxanthin was 0.01μg/injection with a S/N ratio of 26.7 and 5.4, respectively. 0.01μg/injection all-*trans*-retinol was the lowest calibrant standard detected, all samples had levels detected above this detection limit, therefore, S/N was not an issue for carotenoid/retinoid and samples since the injection concentrations were higher in the samples. Plasma samples yielded a 94.9% recovery rate. Plasma α-carotene, β-carotene, lutein, zeaxanthin, and retinol had intra-assay CVs of 2.3%, 7.8%, 0.07%, 3.5%, and 2.8%, respectively.

Enzyme-linked immunosorbent assay of oxidative stress markers

Plasma 8-iso prostaglandin F2α was analyzed using Cusabio (Cat.#CSB-E12100h, Hubei, P.R. China) enzyme-linked immunosorbent assay (ELISA) kits. All protocols were followed as provided and lower limit of detection was 78 pg/mL for 8-iso prostaglandin F2α. Duplicates were run for 8-iso prostaglandin F2α and had intra-assay CVs of 10.3% for 8-iso prostaglandin F2α.

Statistical Analysis

The effect of carrot supplementation was analyzed using nested two-way analysis of variance (ANOVA) with repeated measures using SAS 9.2 (SAS Institute Inc., Toronto, ON). Probability $\alpha \leq 0.05$ was considered statistically different. When no interaction effect of diet and time was identified, the ANOVA was re-tested for the main factor, which enabled us to test against the correct degree of freedom of error. Significant effects of carrot supplementation on ERG measures were analyzed using the paired t-test provided by SAS. A Pearson correlation coefficient was computed to

evaluate the relationship between plasma carotenoids and cholesterol. An absolute value of r with strength of 0.40-0.59, 0.60-0.79, and 0.80-1.0, was described as moderate, strong, and very strong, respectively. Data from one participant in the carrot group and one from the control group was excluded in the Pearson correlation analysis as they had a plasma β-carotene concentration greater than 1 standard deviation from the mean (1.46 ± 0.51). The use of the word "trend" is defined as $P<0.1$. Data is expressed as mean ± standard error of the mean (SEM), unless specified otherwise.

Chapter IV. RESULTS

Basic demographic data

Demographic, anthropometric, and computer use data

Age and gender amongst the two groups were similar (Table 4-1). BMI did not change in either group from PRE to POST measurements (Cont, 24.2 ± 1.1; Carrot, 23.5 ± 1.9) indicating that the supplements had no effect on weight measures (Table 4-1). Five percent (n=1), 68% (n=13), and 26% (n=5) of participants fell within the underweight, normal, and overweight/obese BMI reference ranges, respectively (Table 4-1).

The majority of participants in both groups (58%, n=11) rated their health status as very good, and only 1 (5%) participant perceived their health status as fair. Those of the carrot group used the computer for shorter (4-6 hr/day), more frequent intervals (7 days/wk) (Table 4-1). In comparison, the majority of the control group used the computer for longer (6+ hr/day), frequent intervals (6-7 days/wk) (Table 4-1).

Screening data

The most common reported symptoms amongst screened (n=53) individuals with CVS are listed in Figure 4-1. Eyestrain, eye fatigue, and headache are the top complaints among computer users with 77%, 75% and 59%, respectively (Fig. 4-1). This data corresponds with the top symptoms as reported by eligible participants only.

Table 4-1. Basic demographic, anthropometric, and computer usage data

| Characteristic	Control (n=9)	Carrot (n=10)
Age (yrs)	32.8 ± 3.7	33.2 ± 5.3
Gender		
Male	1	3
Female	8	7
Height (m)	1.67 ± 0.03	1.64 ± 0.02
Weight (kg)	67.9 ± 4.4	63.4 ± 5.6
BMI (kg/m^2)	24.2 ± 1.1	23.5 ± 1.9
<18.5	0	1
18.5-24.9	5	8
25.0-29.9	4	0
≥30	0	1
Health Status (%)		
Fair	0 (0)	1 (10)
Good	5 (56)	2 (20)
Very Good	4 (44)	7 (70)
Computer use hrs/day (%)		
4-6 hrs	4 (44)	7 (70)
6-7 hrs	3 (33)	1 (10)
7+ hrs	2 (22)	2 (20)
Computer use days/wk (%)		
5 days	1 (11)	0 (0)
6 days	3 (33)	1 (10)
7 days	5 (56)	9 (90)

Values expressed as mean ± SEM
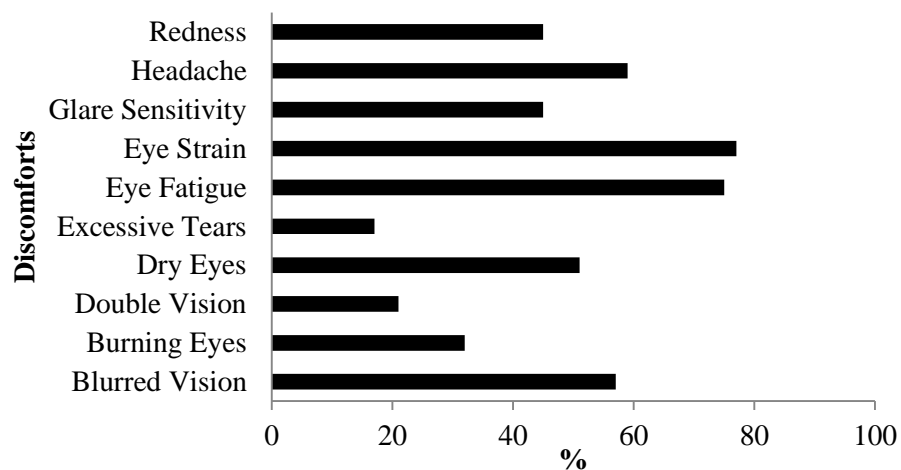
Figure 4-1: Ocular discomforts experienced by chronic computer users. Data expressed as percentage reported (n=53) by all screened participants.

Carrot powder supplementation effects on retina function

ERG measurements were used to evaluate the effects of carrot supplementation on retinal rod and cone function in chronic computer users.

Carrot powder supplementation on dark-adapted responses

Carrot powder supplementation did not significantly alter rod-driven photoreceptor (a-wave) and bipolar cell (b-wave) amplitudes (Fig. 4-2A&B). Although there appears to be a diet effect present in the rod-driven oscillatory potentials, this is due to higher initial oscillatory potentials in the carrot group in comparison to the control group, however, from PRE to POST measurements, there is no difference within the two groups (Fig. 4-2C). No significant differences were seen in rod-driven a- and b-wave implicit times in the both the control and carrot group (Fig. 4-2D&E).

Carrot powder supplementation on light-adapted conditions

Although pictorial data representation illustrates a marginal trending improvement in cone-driven bipolar cell function following carrot supplementation, data did not reach statistical significance (P<0.1) (Fig. 4-3A). Additionally, separation of right and left retinal responses of the carrot group reiterates the diagrammatical data trend improvements of cone-driven b-waves seen in 75% (12/16) of all retinas, compared to only 47% (8/17) in the control group retinas (Fig. 4-3 C & D). Under photopic conditions, a typical b-wave ERG recording following carrot supplementation at PRE and POST time points is shown in Figure 4-3E. Initial cone-driven oscillatory potentials were lower in the carrot group compared to the control, however, both experienced a reduction after 4 weeks without reaching statistical significance (Fig. 4-3B).

Figure 4-2: Effects of carrot powder supplementation on ERG dark-adapted responses. Data expressed as mean ± SEM (n=9-10 participants/group). No statistical differences were identified by paired t-test model. (A) a-wave maximum amplitude; (B) b-wave maximum amplitude; (C) Sum oscillatory potential maximum amplitude; (D) a-wave implicit time at intensity 1.4 log sc cd•sec/m2; (E) b-wave implicit time at intensity 1.4 log sc cd•sec/m2.
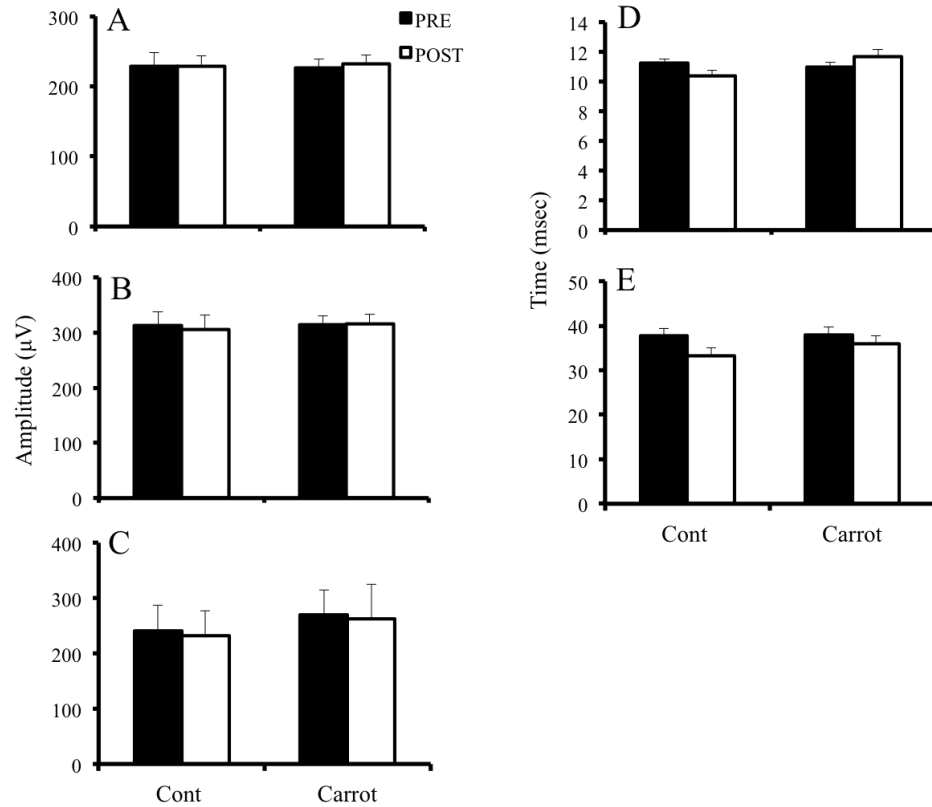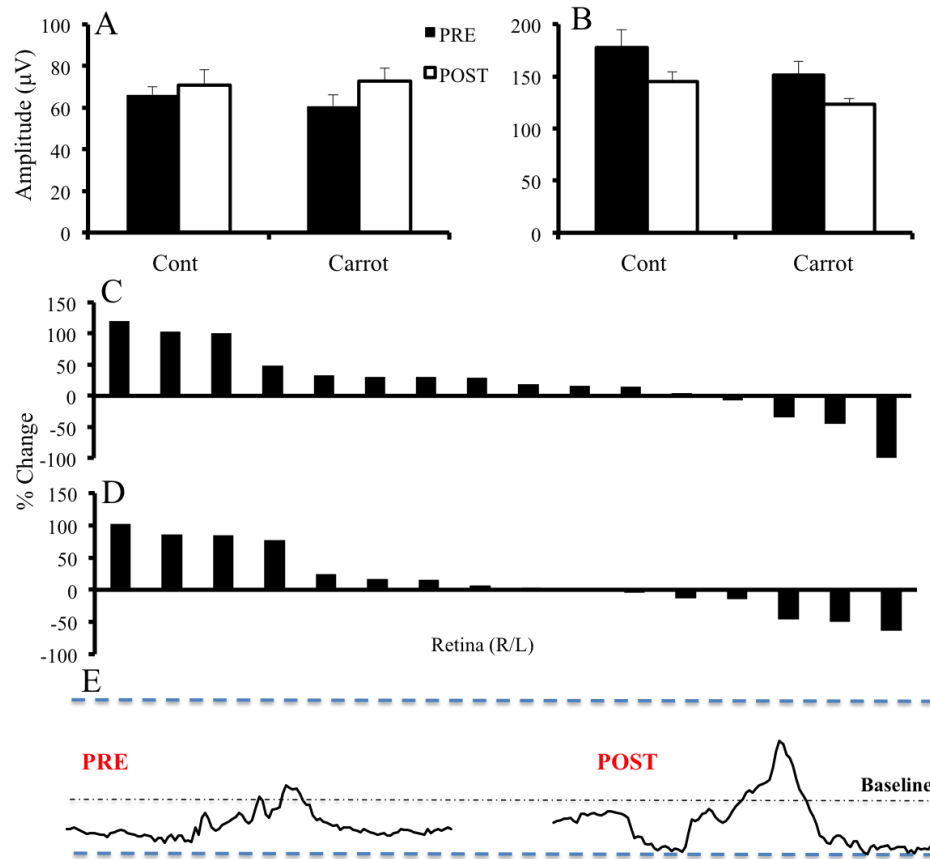
Figure 4-3: Effects of carrot powder supplementation on ERG light-adapted responses. Data expressed as mean ± SEM (n=9-10 participants/group). No statistical differences were identified by paired t-test model. (A) b-wave maximum amplitude; (B) Sum oscillatory potential maximum amplitude (C) b-wave maximum amplitude percent change of individual retinas (R/L) in carrot group; (D) b-wave maximum amplitude percent change of individual retinas (R/L) in control group; (E) Representative ERG of b-wave amplitude at PRE and POST time points following carrot supplementation.

Carrot powder supplementation effects on retinoid and carotenoid status

Retinoid and carotenoid levels were measured in the plasma to examine if carrot powder supplementation, rich in carotenoids, is converted to retinoids and available carotenoids for tissue uptake.

Plasma samples were analyzed for retinol, β-carotene, lutein, and zeaxanthin. Plasma retinol significantly increased from PRE to POST as a function of time in both the carrot

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(17% ± 6.2, 1.29µg/mL ± 0.13) and control (12% ± 6.1, 1.24µg/mL ± 0.10) groups (P<0.01) (Fig. 4-4). Participants fed the carrot supplement had significant and continuous increases in plasma β-carotene percent changes (P<0.02) (Fig. 4-4). No significant differences were found in plasma lutein and zeaxanthin concentrations between groups (Table 4-2).

A strong positive correlation was found after providing carrots between plasma β-carotene and lutein (r = 0.822), compared to the control, which exhibited a moderate correlation (r = 0.566) (Fig. 4-5A). A similar strong positive correlation in the carrot group was seen between plasma β-carotene and zeaxanthin concentrations (r=0.742), but was not seen in the control group (r = 0.124) (Fig. 4-5B). As expected, a moderate negative correlation was associated with plasma β-carotene and retinol (r = -0.484); however, no correlation was seen in the control group (r = -0.007) (Fig. 4-5C). The relationship between plasma β-carotene and lutein and cholesterol concentrations was also assessed to evaluate if carotenoid absorption is associated with plasma cholesterol. A moderate positive correlation exists in the carrot group between β-carotene and LDL (r = 0.559) and lutein and LDL (r = 0.586), but was not reciprocated in the control group (r = -0.355 and -0.111, respectively) (Fig. 4-6).
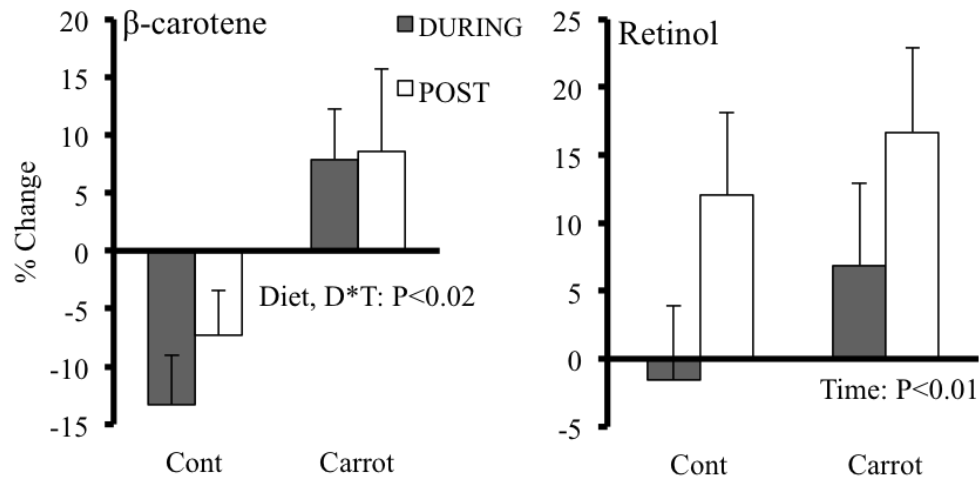
Figure 4-4: Effects of carrot powder supplementation on plasma carotenoids and retinoids. Expressed as average percentage change in 1mL of plasma. Data expressed as mean ± SEM (n=9-10 participants/group). Significant effects of diet and time were identified by nested two-way ANOVA model with repeated measures. Significant diet effects: β-carotene (P<0.02). Significant time effects: retinol (P<0.01). Significant interaction effects: β-carotene (P<0.02).

Table 4-2. Plasma retinoid and carotenoid concentrations at PRE, DURING, and POST time points, in control and carrot groups.

µg/mL	Control (n=9)			Carrot (n=10)			Significant effects		
	PRE	DURING	POST	PRE	DURING	POST	Diet (P<)	Time (P<)	D*T (P<)
Retinol	1.11(0.19)	1.08(0.24)	1.24(0.31)	1.10(0.23)	1.16(0.28)	1.29(0.40)	NS	0.007	NS
β-carotene	1.68(0.69)	1.41(0.51)	1.51(0.50)	1.42(0.77)	1.46(0.51)	1.46(0.52)	NS	NS	NS
Lutein	0.24(0.05)	0.23(0.05)	0.25(0.07)	0.23(0.04)	0.23(0.05)	0.23(0.06)	NS	NS	NS
Zeaxanthin	0.11(0.017)	0.11(0.014)	0.11(0.019)	0.11(0.013)	0.11(0.017)	0.11(0.017)	NS	NS	NS

Values expressed as mean(SD). Significant effects of diet and time were identified with nested two-way ANOVA with repeated measures. NS, not significant

Figure 4-5: Relationship of plasma carotenoids and retinol with plasma β-carotene.
A Pearson correlation coefficient was computed for control (n=8) and carrot (n=9)
groups. (A) Plasma lutein versus β-Carotene (Control y = 0.1024x + 0.1143, $r^2 = 0.320$,
r = 0.566; Carrot y = 0.1519x + 0.0284, $r^2 = 0.676$, r = 0.822); (B) Plasma zeaxanthin
versus β-carotene (Control y = 0.0065x + 0.1041, $r^2 = 0.015$, r = 0.124; Carrot
y = 0.0356x + 0.0628, $r^2 = 0.551$, r = 0.742); (C) Plasma retinol versus β-carotene
(Control y = -0.0058x + 1.2329, $r^2 = 0$, r = -0.007; Carrot y = -0.5492x + 2.0085,
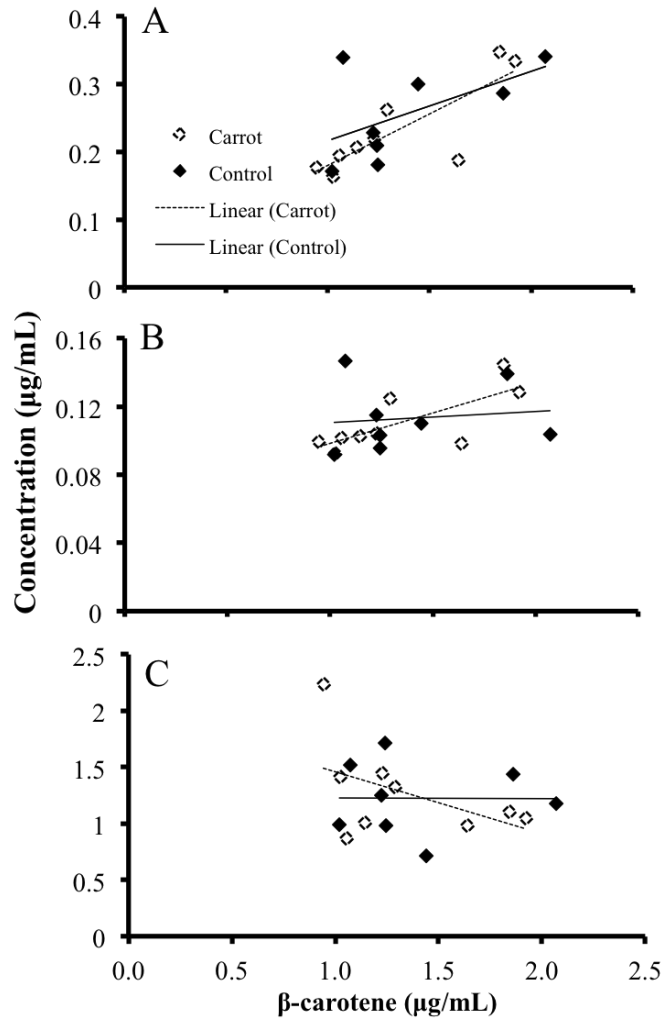$r^2 = 0.234$, r = -0.484).
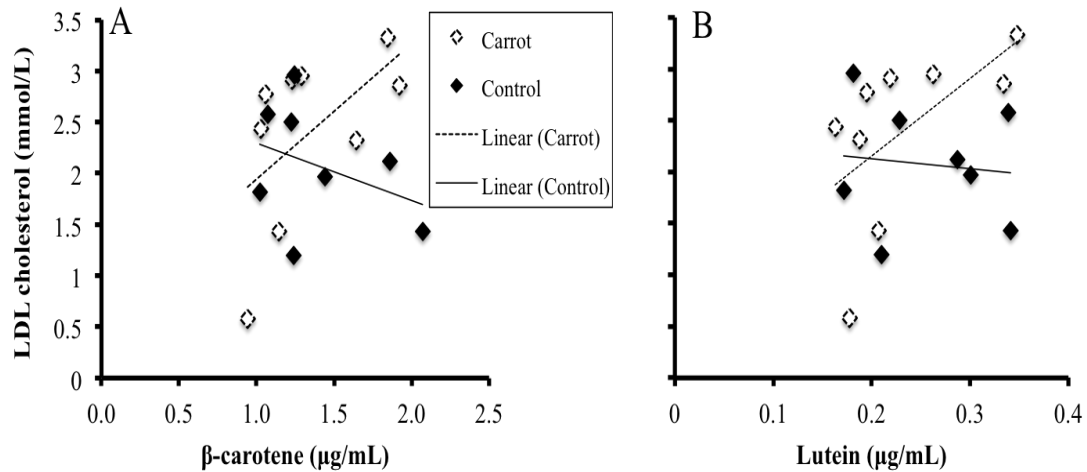
Figure 4-6: Relationship of plasma carotenoids with LDL cholesterol. A Pearson correlation coefficient was computed for control (n=8) and carrot groups (n=9). (A) LDL cholesterol with β-carotene (Control y = -0.5602x + 2.8554, r^2 = 0.126, r = -0.355; Carrot y = 1.3249x + 0.6182, r^2 = 0.313, r = 0.559); (B) LDL cholesterol with lutein (Control y = -0.9685x + 2.3219, r^2 = 0.012, r = -0.111; Carrot y = 7.524x + 0.6497, r^2 = 0.344, r = 0.586).

Carrot powder supplementation effects on plasma lipids and glucose

Lipid parameters and glucose were measured in the plasma to examine the effects of carrot supplementation.

Plasma lipids and glucose

Plasma samples were analyzed for LDL, HDL, VLDL, TC, and TG. Although the carrot group participants had an overall average decrease in LDL cholesterol (-3.2% ± 10.7) compared to the control group (9.2% ± 4.2) after 4 weeks of supplementation, values did not reach statistical significance (Fig. 4-7). HDL concentration increased as a function of time (P<0.04) in both groups (Fig. 4-7). No significant differences were found in plasma TC, TG, and VLDL concentrations between groups (Table 4-3).

Plasma glucose was measured to identify if carrot supplementation resulted in the maintenance of blood glucose concentrations. A significant time change was seen in both the control and carrot groups (P<0.05) (Fig. 4-7).
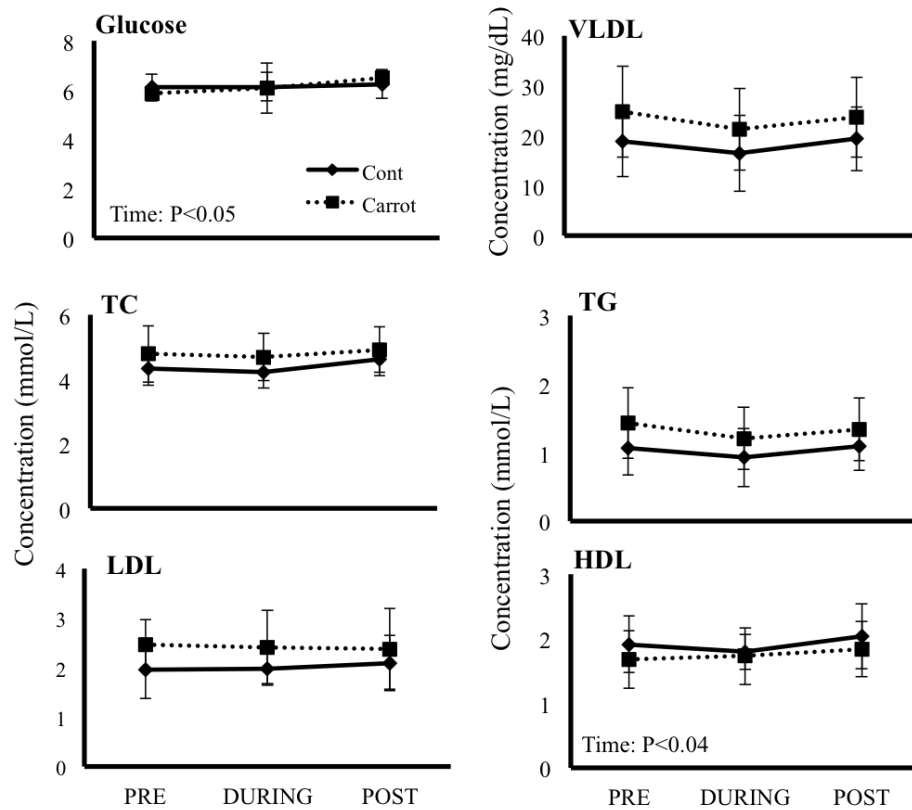
Figure 4-7: Effects of carrot powder supplementation on plasma lipids and glucose. Data expressed as mean ± SEM (n=9-10 participants/group). Significant effects of diet and time were identified with nested two-way ANOVA with repeated measure. Significant diet effects: NS. Significant time effects: Glucose (P<0.05), HDL (P<0.04). Significant interaction effects: NS. HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; TC, total cholesterol; TG, triglyceride; VLDL, very low-density lipoprotein.

Table 4-3. Plasma lipids, glucose and oxidative stress parameters at PRE, DURING, and POST time points, in control and carrot groups.

	Control (n=9)			Carrot (n=10)			Significant effects		
							Diet (P<)	Time (P<)	D*T (P<)
	PRE	DURING	POST	PRE	DURING	POST			
Glucose (mmol/L)*	6.1(0.54)	6.13(0.57)	6.24(0.59)	5.87(0.27)	6.09(1.02)	6.48(0.27)	NS	0.05	NS
TC (mmol/L)	4.34(0.54)	4.22(0.50)	4.61(0.50)	4.78(0.87)	4.69(0.74)	4.92(0.71)	NS	NS	NS
LDL (mmol/L)	1.95(0.58)	1.80(0.59)	2.09(0.56)	2.46(0.52)	2.40(0.76)	2.38(0.82)	NS	NS	NS
HDL (mmol/L)	1.91(0.44)	1.80(0.27)	2.03(0.51)	1.68(0.45)	1.73(0.44)	1.84(0.23)	NS	0.04	NS
VLDL (mg/dL)	18.89(7.08)	16.50(7.62)	19.33(6.32)	25.2(8.69)	21.3(8.19)	27.1(13.25)	NS	NS	NS
TG (mmol/L)	1.07(0.39)	1.34(1.31)	1.09(0.35)	1.43(0.49)	1.21(0.48)	1.53(0.74)	NS	NS	NS
F2-Isoprostanes (pg/mL)	0.043(0.009)	0.041(0.007)	0.043(0.01)	0.044(0.007)	0.044(0.009)	0.043 (0.009)	NS	NS	NS

*Carrot group (n=9) in glucose analysis. Values expressed as mean(SD). Significant effects of diet and time were identified with nested two-way ANOVA with repeated measurement. HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; TC, total cholesterol; TG, triglyceride; VLDL, very-low density lipoprotein.

Carrot powder supplementation effects on plasma oxidative stress markers

Oxidative stress markers were measured in the plasma to examine if they are influenced by carrot supplementation. Plasma F2-Isoprostane concentration showed a reduction trend from PRE to POST time points following supplementation, however values did not reach statistical significance (P<0.1) (Fig. 4-8).
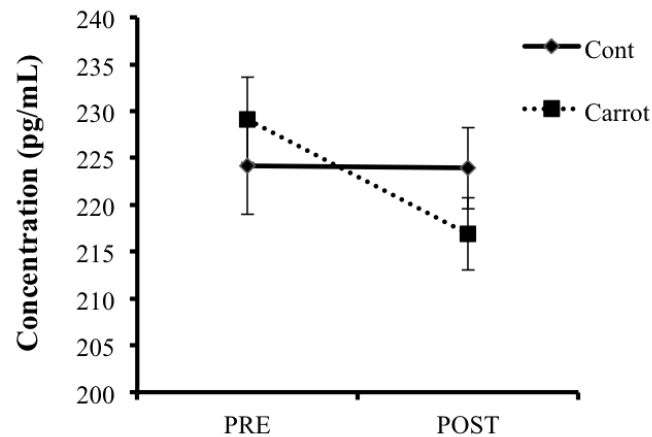
Figure 4-8: Effects of carrot powder supplementation on plasma oxidative stress marker, F2-isoprostane. Expressed as mean ± SEM (n=9-10 participants/group). No statistical differences were identified by nested two-way ANOVA with repeated measures (P<0.1).

Carrot powder supplementation effects on CVS symptoms

Self-perceived CVS symptoms were assessed to examine if carrot supplementation improves participants self-reported symptoms. Carrot supplementation did not alter self-perceived symptoms (Fig. 4-9).
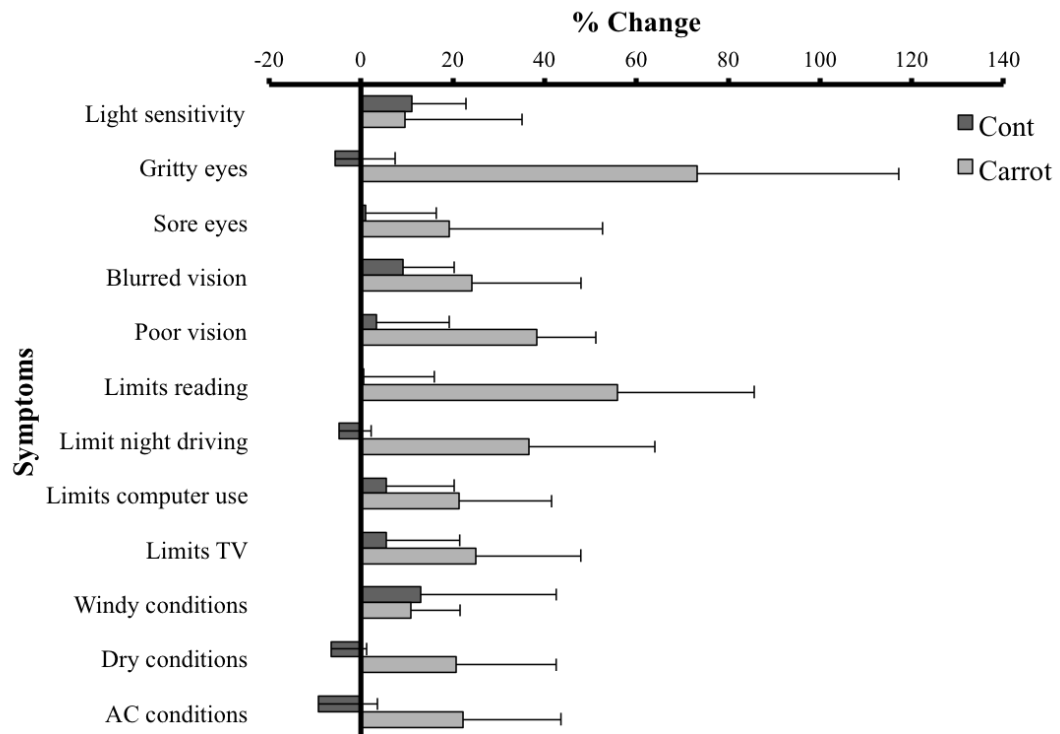
Figure 4-9: Effects of carrot powder supplementation on CVS symptoms. Expressed as mean ± SEM (n=9-10 participants/group). Carrot powder supplementation did not alter self-perceived symptoms of CVS.

Chapter V. DISCUSSION

This is the first study to examine the effects of carrot powder supplementation on retinal function in long-hour computer users measured by ERG responses. Carrot supplementation produced a non-significant trend (P<0.1) improving cone-mediated inner retina function in 75% of total retinas, while continuously and significantly increasing plasma retinol and β-carotene during the 4-week supplementation period. The increase in vitamin A and carotenoids, however, was not associated with ocular related symptom improvement. Carrot supplementation played a protective role on blood lipids by significantly increasing plasma HDL cholesterol as a function of time (P<0.04) along with displaying a LDL cholesterol-lowering pattern, without reaching statistical significance. A strong positive correlation between plasma β-carotene and lutein with LDL cholesterol was seen (r = 0.559 and 0.586, respectively) in the carrot group. Lastly, a clear positive relationship of plasma β-carotene with plasma lutein and zeaxanthin was exhibited (r = 0.822 and 0.742, respectively), and a moderate negative correlation between plasma β-carotene and retinol (r = -0.484) was identified following carrot supplementation, supporting genetic variability in carotenoid metabolism. These results indicate that carrot supplementation, in the amount of 33% of the vitamin A RDA for adults, has the potential to improve retina function and blood lipid profiles in CVS in as little as 4 weeks.

Carrot supplementation on retina function

Retina function is known to be affected by nutrients supplemented in the diet; however, to date this is the first study to examine the effects of carrot supplementation on retinal function measured by ERG response. This present study found that healthy participants with long-hour computer use had marginally improved cone-mediated inner retina function following carrot supplementation suggesting improved phototransduction, although data did not reach statistical significance. Comparatively, scotopic (rod-driven)

a- and b-wave amplitudes were not altered. Similar pronounced effects to cone cells compared to rod cells has been reported in the literature. Ventura and associates (2004) found greater detrimental effects to cone cells than rod cells in people exposed to mercury vapor, whilst Bartel et al. (2000) reported abnormal b-waves and normal a-waves in HIV-positive individuals. Similarly, β-carotene supplementation improved ERG b-wave responses by 40% from baseline in individuals with retinitis pigmentosa (Rotenstreich et al., 2013). Furthermore, in an animal study performed in our laboratory, carrot supplementation induced greater improvements to rodent b-wave responses (McClinton et al., 2014), highlighting the sensitivity of neural cells. While the mechanisms of the differential effects on photoreceptors and inner retinal layers are not known, the improvements seen in this study may be related to the increased availability of retinol and carotenoids within the plasma, due to carrot supplementation, ultimately for use within photoreceptor cells.

Since cone cells are highly enriched in the macular region, an area designed for visual acuity and blue light absorption, improvement to photopic ERG responses following carrot supplementation seen in this study supports recent evidence on deposition and preferential uptake of carotenoids in the macula lutea (Li et al., 2014; During et al., 2008). Another explanation for photopic improvements could be based on findings from Wang et al. (2009) that upon isolation of neural retinas from the RPE, rod cells were unable to regenerate following a light stimulus, however, cone cells maintained the ability to regenerate. This indicates a discrete recovery cycle for cones independent of the RPE (Wang et al., 2009; Wang & Kefalov, 2009), suggesting that cones require a greater quantity of 11-*cis*-retinoids (Ala-Laurila et al., 2009). Regeneration of 11-*cis*-retinal is believed to occur within the RPE exclusively, however, recent work suggests that cone cells also acquire 11-*cis*-retinoids from Müller cells (Mata et al., 2002; Muniz et al., 2007). Unlike rods, cones contain a yet-to-be-specified retinal oxidoreductase capable of

oxidizing 11-*cis*-retinol to 11-*cis*-retinal, facilitating pigment regeneration (Ala-Laurila et al., 2009). The process by which 11-*cis*-retinol is transferred preferentially to cone cells from Müller cells remains contentious, along with other molecular components of the cone visual cycle. Using *irbp*$^{-/-}$ knockout mice, cone response saw a 50% reduction, illustrating that cone function is more affected by the ablation of IRBP than rod cells (Jin et al., 2009). It is thus plausible that IRBP displays an affinity towards the cone matrix sheath, allowing cones direct and efficient access to 11-*cis*-retinoids and removal of all-*trans*-retinol (Garlipp et al., 2012), facilitating a greater cone response when combined with an abundance of vitamin A, as seen in our study. The full beneficial mechanistic action of carotenoids on visual function remains to be elucidated.

This study targeted generally healthy, and relatively young, long-hour computer users. Unlike cone cell function, carrot supplementation did not alter scotopic function after 4 weeks. Supplementation of isolated carotenoids to individuals with retinitis pigmentosa, age-related retinal diseases, and vitamin A deficiencies, has displayed improvements in scotopic function, however, baseline values were nearly three-fold less in comparison to baseline values obtained in our study (Rotenstreich et al., 2013; McBain et al., 2007; Rotenstreich et al., 2010). Therefore, it seems likely that the copious availability of retinoid compounds within the retina, paired with lower baseline retina function, resulted in significant advantageous effects in those studies. In comparison to previous studies, which supplemented isolated carotenoids, this study provided carotenoid supplementation in whole food form. Whole foods contain the less stable, all-*trans*-β-carotene form versus the more stable, 9-*cis*-β-carotene form present in supplements (Rotenstreicht et al., 2010) and thus, theoretically, can yield superior quantities of rhodopsin within photoreceptor cells (Rotenstreicht et al., 2010). Considering carotenoid forms affect the absorption and conversion to vitamin A differently, our carrot powder, providing 33% of the vitamin A RDA, may be insufficient

to improve scotopic measurements in healthy individuals. Future studies should include a dose response paradigm to clarify the full potential of the carrot on visual function.

Due to the relatively new nature of CVS, retinal function of those with CVS symptoms or diagnoses, have yet to be assessed or reported. As such, this study provides insight into retina function in these individuals, indicating that long-hour computer users do not have any apparent retinal dysfunctions. However, the mean age of participants was fairly young, at approximately 33 ± 13.9 years old, therefore, it would be interesting to examine retinal function of an average older population, with a longer period of computer use. As previously noted, recent research indicates that detrimental ocular effects of blue light exposure from VDTs to RPE and photoreceptor cells is chronic rather than acute, possibly creating retinal problems in the latter years (Nowak, 2013; Hunter et al., 2012). As such, we should not be hasty to dispel retinal dysfunction associated with computer use, as this issue remains unknown.

Carrot supplementation on plasma vitamin A and carotenoid concentrations

Plasma concentrations of vitamin A and carotenoids were measured to assess the impact of carrot supplementation and determine the trend of plasma concentrations throughout the course of 4 weeks. Participants fed the carrot supplement had significantly higher β-carotene concentrations than their control group counterparts. The concentration was continuously increased from baseline to the week 2 and further week 4 time points. No significant differences were seen in plasma lutein and zeaxanthin concentrations. Perhaps a longer-term study is needed to understand the saturation level of carotenoid supplementation within the plasma. As anticipated, due to the increase in plasma carotene concentrations, retinol plasma concentrations subsequently increased as a function of time in the carrot group. However, retinol concentrations in the control group also increased as a function of time, which cannot be explained by the dietary treatment given.

This may be attributed to the result of conducting a free-living trial where the diets of participants were not controlled for foods rich in carotenoids and vitamin A, although the initial instruction was given to refrain from such foods. Future studies should include a dietary recall to gain a more accurate picture of nutritional status, with a controlled-feeding trial being the optimal study design to minimize variability between dietary intakes.

Significant inter-individual carotenoid absorption variability has been exhibited in both men and women, indicating a potential genetic factor independent of nutritional status (Lin et al., 2000; Hickenbottom et al., 2002; Clark et al., 2006). Our data supports this variability, and further, presents a strong positive correlation with plasma β-carotene and lutein ($r = 0.822$) and zeaxanthin ($r = 0.742$) concentrations, compared to the control group ($r = 0.566$ and $r = 0.124$, respectively). Comparatively, there was a moderate negative correlation ($r = -0.484$) between plasma β-carotene concentrations and retinol, in comparison to the control group ($r = -0.007$), which theoretically supports the speculated interindividual variability mechanism of intestinal β-carotene conversion following supplementation (discussed below) (Borel et al., 1998). As such, those exhibiting polymorphisms in the BCMOI and BCDOII genes will have hindered conversion of β-carotene to retinol, resulting in high plasma β-carotene and low plasma retinol. Numerous investigators have reported differing responses between individuals following a supplementation dose (Borel et al., 1998; Brown et al., 1989; Nierenberg et al., 1991), and this variability is further supported by data from our study. Although the precise mechanism behind interindividual variability remains unknown, three speculative mechanisms have been proposed (Borel et al., 1998). The first plausible mechanism aiding in interindividual variability is the rate of intestinal β-carotene absorption (Borel et al., 1998; Hickenbottom et al., 2002). The differing absorption rate may be due to single nucleotide polymorphisms (SNPs) in genes associated with lipid metabolism, such as SR-

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BI and ABC transporters, ultimately affecting carotenoid absorption and transport, thus, influencing plasma concentrations (Borel, 2012; Herbeth et al., 2007; Borel et al., 2007). The second mechanism is the variation in efficacy of intestinal conversion of β-carotene to vitamin A (Borel et al., 1998). The reason for the variability in conversion efficiency appears to be related to numerous polymorphisms in the BCMOI and BCDOII gene, resulting in hindered cleavage (Lietz et al., 2012; Hendrickson et al., 2012; Leung et al., 2009). The third mechanism is via lipoprotein metabolism, which may influence β-carotene response as it acts as the main form of transport (Shete & Quadro, 2013). In particular, genes that encode for apolipoproteins, LPL, and pancreatic lipase have been associated with varying plasma carotenoid levels (Borel et al., 2007; Herbeth et al., 2007; Borel et al., 2009). Furthermore, Clark and colleagues (2006) have evaluated the association of carotenoid levels with hypo- and hyper- cholesterol responders since cholesterol acts as the main carotenoid transporter. They found a strong positive association between the plasma response to dietary cholesterol and lutein and β-carotene, with plasma lutein more consistently correlated to dietary cholesterol than β-carotene (Clark et al., 2006). The results of our study support the evidence from Clark and colleagues (2006) with moderate associations identified between β-carotene and lutein with LDL cholesterol in the carrot group. Comparatively, a negative association was seen in the control group between LDL cholesterol with β-carotene ($r = -0.355$) and lutein ($r = -0.111$). Although much remains unknown, it is evident that intervariability exists due to genetic differences amongst the population. This present study warrants further investigation into the underlying mechanisms and genetic variants associated with low and high responders with a larger sample size.

Although retinol and carotenoid concentrations increased following supplementation, all reported plasma vitamin A concentrations and carotenoid concentrations were within the upper end of reference ranges 0.3-1.2 μg/mL and 0.5-2

μg/mL, respectively, as based on previously reported values in the literature (Aleman et al., 2013; Mahan et al., 2012). This is despite using varied extraction procedures and chromatographic conditions, since no standard procedure exists within the research community The dose of carrot supplementation given was only 15g/day or 29% and 37% of the RDA for males and females, respectively. The dosage was therefore not a saturation of the RDA but rather only 1/3 of it, acting as an obtainable amount to access in the diet and allowing for retinol and carotenoid plasma concentrations to increase further, as indicated in our data. This amount of carrot powder was chosen due to research identifying pro-oxidant toxic byproducts following excess carotenoid metabolism. As previously mentioned, the retina is equipped with numerous defense lines, such as lipofuscin, to combat the oxidative damage generated from ROS, however, specific byproducts of these defenses may result in deleterious effects (Hunter et al., 2012; Nowak, 2013). The lipofuscin constituent, N-retinaldehyde-N-retinylethanolamine (A2E) – a bisretinoid – is capable of absorbing blue light wavelengths whilst concomitantly producing molecules able to induce injury to the RPE (Wu et al., 2010; Zhou et al., 2006). Once a critical intralysosomal concentration of A2E is attained, A2E is released from the lysosome where it may then bind outer mitochondrial membranes, ultimately inducing RPE apoptosis (Schutt et al., 2000). Although not tested, it is plausible that due to the increased presence of retinoid molecules available for utilization within the retina following high dose chronic supplementation, there may be an overt production of bisretinoids, leading to detrimental effects. This evidence supports the minimal supplement dose provided in this study.

The transport of carotenoids on chylomicrons may also influence analyzed plasma carotenoid levels. Analyzed plasma was obtained under 12 hour fasted conditions, therefore lacking chylomicrons, and thus possibly influencing the representation of plasma vitamin A derivatives and carotenoid concentrations. Under fasted conditions, up

to 75% of β-carotene is found in LDL, and to a lesser extent HDL and VLDL, respectively (Yeum & Russell, 2002). It has been shown that the polar carotenoids, such as lutein and zeaxanthin, accumulate in the core of the chylomicrons and chylomicron remnants. Therefore, future studies should also include post-prandial blood collection to obtain insight into the incremental increases in plasma carotenoid and retinoid concentrations, following ingestion of supplementation.

Carrot supplementation on plasma oxidative stress markers

Since carotenoids act as antioxidants possessing the ability to reduce oxidative damage caused by ROS we measured oxidative stress markers in the plasma to determine the effects of carrot supplementation. Despite the increased plasma carotenoid concentrations following carrot supplementation, only a trending decrease in plasma markers of oxidative stress (F2-isoprostanes) was seen without reaching statistical significance. Our findings are consistent with those of Briviba et al. (2004), which demonstrated no effect of 330mL carrot juice (27mg β-carotene, 13mg α-carotene) on lipid peroxidation biomarkers in plasma and feces of healthy men. The lack of change in oxidative stress is also seen in a study conducted by Crane et al. (2011) following a diet high in carotenoid rich fruits and vegetables. These results do, however, contradict an intervention trail by Butalla et al. (2012) following a 3-week carrot juice dose-response study in overweight breast cancer survivors that observed a 87% reduced odds ratio of increasing 8-iso-PGF2α levels for participants in the highest carotenoid change range compared with those in the lowest range. Numerous studies have demonstrated that breast cancer patients exhibit higher concentrations of oxidative stress than healthy individuals, which could indicate that carotenoid concentrations are only protective in diseased bodies with high concentrations of oxidative stress, and not in healthy individuals (Butalla et al., 2012). It is plausible values did not reach statistical significance due to the limited sample size and length of study needed to vastly affect

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oxidative stress levels. Due to mixed evidence, the effect carrot supplementation has on oxidative stress markers needs to be examined in a larger sample size to substantiate a conclusion.

Carrot supplementation on plasma lipid and glucose concentrations

The anti-hypercholesterolemic effect of dietary fiber is well established, however, scarce evidence exists on the cholesterol effects of complex vegetables, such as carrots. Carrots are a rich source of fiber; 4 grams of carrot powder found in the supplements of this study provide almost 20% of the total dietary fiber recommendations, with soluble fiber representing 8% to 50% of total fiber (Arscott and Tanumihardjo, 2010). Therefore, plasma lipid and glucose levels were measured to assess the impact of carrot supplementation and determine the trend of plasma levels throughout the course of 4 weeks. During this study, HDL cholesterol significantly increased as a function of supplementation period in both the control and carrot group, along with a reduction trend in LDL cholesterol following carrot supplementation. Although carrot supplementation beneficially improved HDL cholesterol levels, this cannot be attributed to the fiber present in the carrot as similar results were seen in the control group. While the control supplement did not contain any fiber, the increase in HDL cholesterol could be due to other external dietary factors as a result of conducting an *ad lib* study.

Suggestively, the LDL cholesterol-lowering trend seen in our results appears to follow and substantiate the established anti-hypercholesterolemic mechanism of dietary fiber, through the binding and excretion of bile acids. A study conducted by Nicolle and colleagues (2003) saw no reductions to fecal bile acid losses, confirming that the carrot does not act by reducing cholesterol absorption. Interestingly, the role of fermentation end-products, collectively known as short-chain fatty acids (SCFA) such as butyrate, are notably involved in the role of fiber on lipid metabolism (Nicolle et al., 2003). Butyrate

concentrations, which significantly increased (123%) following carrot intake, were demonstrated using a Caco-2 cell line to regulate apoA-IV secretion, resulting in a reversal of cholesterol transport causing a cholesterol efflux (Nazih et al., 2001). In addition to the fiber content, carotenoids may also contribute to the carrot's cholesterol-lowering effect. Following consumption of 125mg of β-carotene/kg of diet, hypertensive rats fed a cholesterol rich diet had significantly reduced plasma lipoprotein cholesterol (LDL and VLDL) levels (Tsai et al., 1992). Similar effects were also present in normotensive rats following dietary β-carotene supplementation (Amen & Lachance, 1974). A more recent study conducted by Silva and colleagues (2013) investigated whether β-carotene supplementation has a direct alteration on the cholesterol metabolism genes in hypertensive Fisher rats. The results of this study found no significant differences in genetic changes following supplementation (Silva et al., 2013). Although post-transcriptional regulation of gene-expression is still possible, the results from this study suggest the decreased cholesterol absorption seen by β-carotene is a result of its actions in the intestinal lumen, rather than through alteration of gene expression (Silva et al., 2013). While the lipid-lowering mechanism of carrots is unknown, this study provides further evidence supporting the beneficial outcome of whole carrots as a nutraceutical.

Plasma glucose concentrations were measured as a previous animal study conducted in our laboratory found that a carrot enriched diet had no effect on healthy animals (McClinton et al., 2014). The present study found a significant time difference between, however, no diet effect was seen corresponding to the animal study. The time effect may be a result of conducting a free-living study. Comparatively, a similar study conducted on Wistar rats with metabolic syndrome, saw improved oral glucose tolerance from isolated β-carotene supplementation (Poudyal et al., 2010). These results indicate that the fiber content may not be responsible for glucose handling, but rather a consequence of mechanistic actions of β-carotene.

Carrot supplementation effects on CVS symptoms

Due to the relatively new nature of CVS, little is known whether nutrients supplemented in the diet can provide relief of CVS symptoms. Thus, participants' self-perceived symptoms were analyzed in this study to examine the effects of carrot supplementation on CVS complaints. Although positive effects were seen in photopic inner retinal function, CVS symptoms were not altered. Limited data exists in this field; however, the available studies do elicit positive results for nutritional interventions on CVS related symptoms. One study found that supplementation of sea buckthorn oil attenuated tear film osmolarity and alleviated dry eye symptoms, possibly attributed to the dietary fatty acid composition of the oil; however, the carotenoids present in the sea buckthorn oil may also aid mechanistically, as they exhibit anti-inflammatory effects demonstrated by *in vivo* studies (Larmo et al., 2010; Järvinen et al., 2011; Salminen et al., 2008). Kim and associates (2009) demonstrated improved symptom scores and tear film break-up times in individuals with dry eyes, following vitamin A eye drops. These studies illustrate the potential carrot powder holds to provide relief to long-hour computer users. Since participants of this study did not include any individuals with pre-existing dry eye conditions (kerato-conjunctivitis sicca), it may thus be beneficial to examine the effects of carrot powder in individuals with dry eye syndrome, based on results of previous studies. In this study, the OSDI questionnaire was very vague and subjective as individuals have varying sensitivities to symptoms and stimuli, which could skew the results of the questionnaire. A more descriptive and precise questionnaire should be utilized in future studies, along with confirmation of symptoms by an expert in the field in order to gather the most objective measurement. A larger trial is needed to draw definite conclusions on the therapeutic role of the carrot for CVS symptoms.

Summary

The research conducted in this pilot study ensued exploration into the effects of whole food carrot supplementation on plasma carotenoid/retinoid concentrations and retinal function, enabling examination of retinoid/carotenoid concentrations in conjunction with ERG recordings. Although average plasma carotenoid levels increased following supplementation, interindividual variability exists in plasma retinol, lutein and β-carotene levels, suggestive of genetic underlays inducing hypo- and hyper- responders to carotenoids. Carrot powder supplementation marginally increased photopic b-wave amplitudes in 75% of retinas at only 33% of the vitamin A RDA of adults without statistical differences, however, did not affect scotopic responses. As such, it substantiates evidence suggesting an alternative cone visual cycle, which has never been assessed following supplementation. The transporters involved in the preferential delivery of retinoids from cone cells to Müller cells should be explored and confirmed in a future project. Conversely, although retinal function improved, symptoms associated with CVS were not altered. Therefore, ophthalmologic input regarding confirmation of symptoms and other retinal diseases in participants, in a more objective way, would be beneficial to eliminate the subjective nature of responses. Additionally, retinal function of chronic computer users with CVS appears to be within normative reference ranges, indicating that retinal function is not affected by prolonged computer use nor is retinal function initiating CVS symptoms. Further research should be conducted to highlight the role of carotenoid supplementation in other eye related diseases as a novel nutritional therapy.

Strengths & limitations

This is the first known study to assess retinal function in response to whole carrot supplementation in healthy, chronic computer users. The design of this study was utilized

to examine the effects of carrot powder carotenoids on retinal function in healthy individuals. A dietary amount of whole carrot powder supplemented in our study provides an obtainable and holistic dietary model to the general population that is representative of the average amount of carotenoid intake within our population, allowing for results indicative of normative dietary metabolism. Additionally, a major strength of this study was the development of a simultaneous carotenoid and retinoid analysis technique using UPLC with a C30 column. This methodology allowed us to detect and quantify retinoids and carotenoids in our samples through a single run-time of less than 40 min. As there is no concrete method for identification and quantification of retinoids and carotenoids, it presents a great difficulty for comparison of work between authors. Therefore, presentation of our UPLC carotenoid methodology would be beneficial to the research community to eliminate separate analysis of carotenoids and retinoids, as well to systematically compare research results between authors.

Numerous limitations are present within our pilot study design. Firstly, while the use of whole food allows for a more accurate depiction of its effects within the population, it also makes it difficult to eliminate the possibility that one or multiple compounds of the whole food may have produced our results. The results of this study were assumed to be as a result of the carotenoid content of the carrot powder; however, multiple other compounds are present in the powder but were not examined. Additionally, as this was a pilot study, the sample size was small resulting in a reduced power and thus possibly, impacting statistical significance. However, it is still promising that 75% of total retinas had improved inner neural cell cone function. A larger sample size is needed in order to obtain robust data. There was also an uneven distribution of males and females amongst the two groups, which may affect the results. Female body system interactions with vitamins are more complex due to the presence of sex hormones

estrogen and androgen, which have been shown to affect BCMO gene expression and organ specificity (van Helden et al., 2013).

As previously mentioned, this was a free-living study allowing participants to continue with their normal dietary practices, which could have impacted the results of this study, such as increases in plasma retinol in both the control and carrot group. As such, other than consumption of fish oil pills and multivitamins, participants were not restricted from consuming nutrients that are known to affect visual function. No dietary records were collected, such as a food-frequency questionnaire, which could aide in identifying vitamin A and carotenoid consumption patterns prior to and during the study, as well, identify other nutrients present in their diet that may play a critical role in study results. Additionally, the OSDI questionnaire used by participants to rate their symptoms was very vague and subjective. Individuals have varying sensitivities to symptoms and stimuli, which could skew the results of the questionnaire. It would be beneficial to develop a more descriptive and precise questionnaire to utilize. Ultimately, confirmation of symptoms by a professional and expert in the field, such as an ophthalmologist, would act as the ideal method to obtain CVS symptom data.

Lastly, as a free-living study, the amount of computer use was not regulated between and within each participant, which could illustrate variations between visual function measurements. It is also important to consider other electronic sources in their contribution in CVS symptoms such as smart-phones, handheld tablets, and video game consoles, which were not directly specified to participants to be included in their computer use data. Additionally, ERG recordings were taken throughout the day therefore, the responses of a participant conducted in the morning, prior to any electronic stimuli, may be vastly different than the responses of those conducted in the late

afternoon, after several hours of electronic stimuli. Recordings of each participant should be kept consistent between PRE and POST measurements to minimize variability.

Recommendations for future research

In order to obtain a greater and conclusive understanding on the effects of carrot powder supplementation on healthy, chronic computer users, there are numerous facets that must be explored. The first recommendation is to obtain a larger sample size and conduct a similar study in order to garner more robust data. With a greater sample size, ultimately, a full-feeding trial, with appropriate caloric and nutritional composition between participants, would be ideal in order to eliminate external factors influencing carotenoid status and other parameters. Furthermore, a crossover study paradigm would be beneficial to reduce cofounding variables as each participant serves as his/her own control. However, as this is a large, and costly endeavor, a food-frequency questionnaire and diet restrictions on compounds known to alter visual function, should at least be inaugurated to gain a greater understanding of the dietary patterns of participants. Diet restrictions/minimizations should include, foods rich in β-carotene, vitamin A, xanthophylls, long- and very-long chain polyunsaturated fatty acids, and vitamin/antioxidant supplements.

The second recommendation would be to conduct a dose response study to assess the maximal effect of carrot powder supplementation on visual function measured by ERG responses. The amount of carrot powder utilized in this study was adopted from a previous paradigm conducted in our laboratory (McClinton, 2012), as well based on the negative pro-oxidative effects exhibited following β-carotene supplementation (Schutt et al., 2000; Nowak, 2013; Liu et al., 2009; Fiedor and Burda, 2014). The amount of carrot powder provided represents approximately 33% of the vitamin A RDA for adults, and thus has the potential to increase daily vitamin A intakes. Although our plasma

carotenoid and retinoid data illustrates a positive linear trend, previous studies have reported a saturation mechanism following administration of high β-carotene doses possibly due to limitations in capacity of the plasma transport system (Thürmann et al., 2002; Nierenberg et al., 1991; Woutersen et al., 1999). Therefore, identification of the level in which this occurs must be identified and confirmed through a dose-response paradigm. Conversely, the influence of vitamin A status on carotenoid absorption and utilization must also be taken into consideration when assessing the results. Goswami and colleagues (2003) evaluated the conversion of β-carotene to retinol in Sprague-Dawley rats of varying vitamin A status and found no significant differences in absorption or conversion. As such, further research involving hypo-, normo- and hyper- vitaminosis A humans is required to identify if similar results are replicated in humans and to provide an enhanced understanding of carotenoid metabolism and its influence on visual function.

As this is the first study to assess retinal function in response to carrot carotenoid supplementation, further research should consider if improved ERG amplitudes are correlated with visual acuity, possibly aiding in amelioration of CVS symptoms. Furthermore, as we have seen overall retinal functions improve following supplementation, we now must conduct a multifocal ERG to obtain separate responses across different retinal locations to identify the improved locale.

Lastly, due to the promising results seen from this study on healthy individuals, future research should consider evaluating the effects of carrot powder on other eye related diseases such as diabetic retinopathy and AMD. Carotenoids are a common dietary intervention prescribed to individuals with AMD, however, mixed results concerning this supplementation exists (AREDS2 group, 2013; Murray et al., 2013; Weigert et al., 2011). Although carotenoids are known to possess anti-oxidant capabilities, they have also been shown to elicit pro-oxidant functions under hyperoxic

and imbalanced intracellular conditions, such as AMD and diabetic retinopathy (Kalariya et al., 2009). Under such conditions, carotenoids generate highly reactive oxidized products such as epoxides, ionones, and aldehydes (Kalariya et al., 2009). The role of these oxidation end products remains to be elucidated, however, they have demonstrated cyto- and genotoxic effects at physiological or higher concentrations *in vitro* (Hurst et al., 2005). Kalariya et al. (2009) demonstrated DNA damage to RPE cells following exposure to carotenoid breakdown products (lutein and β-apo-8-carotenal derived bi-products), ultimately contributing to retinal dysfunction. These facts are consistent with results obtained from an animal study conducted in our laboratory assessing the effects of carrot powder on diabetic retinopathy (McClinton et al., 2014). Rats fed the carrot powder had significantly lower rod- and cone- driven retinal function in a STZ-induced Type 1 diabetes model (McClinton et al., 2014). Thus, it is crucial to fully understand the role of carotenoid oxidation products under high levels of oxidative stress with regard to visual function prior to suggesting carrot powder as a novel nutritional therapy for AMD and diabetic retinopathy.

Conclusions

At only one-third of the vitamin A RDA, trending improvements in photopic inner retinal function were seen, possibly as a result of increased plasma carotenoid and retinoid concentrations, indicating retina sensitivity to diet supplementation. As this is the first study examining influence of carrot carotenoids on visual function, future studies should consider multifocal ERG examinations to localize improvements across the whole retinal mass. At present, the underlying mechanism pertaining to a cone visual cycle remains contentious, however, cone-driven improvements seen in this study, supports the research for a separate cone visual cycle requiring ample vitamin A supply. Furthermore, results of this study support interindividual plasma variability in response to carotenoid

supplementation, as seen in previous studies, reiterating the need for further validation of genetic variants involved in carotenoid metabolism. Improvements in lipid parameters may be attributed to the soluble fiber or carotenoid content present in carrots, which can thus act as a dietary cholesterol-lowering treatment. Additionally, retinal function of chronic computer users appears to be within normative reference ranges, however, long-term effects of blue light exposure on ocular tissues should be monitored. Considering the trending improvements seen in cone-driven inner retinal responses, along with increased plasma carotenoid/retinoid levels and beneficial lipid changes, minimal supplementation of carotenoids by carrot powder can be recommended as a novel nutritional therapy for healthy, chronic computer users.

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Appendix A

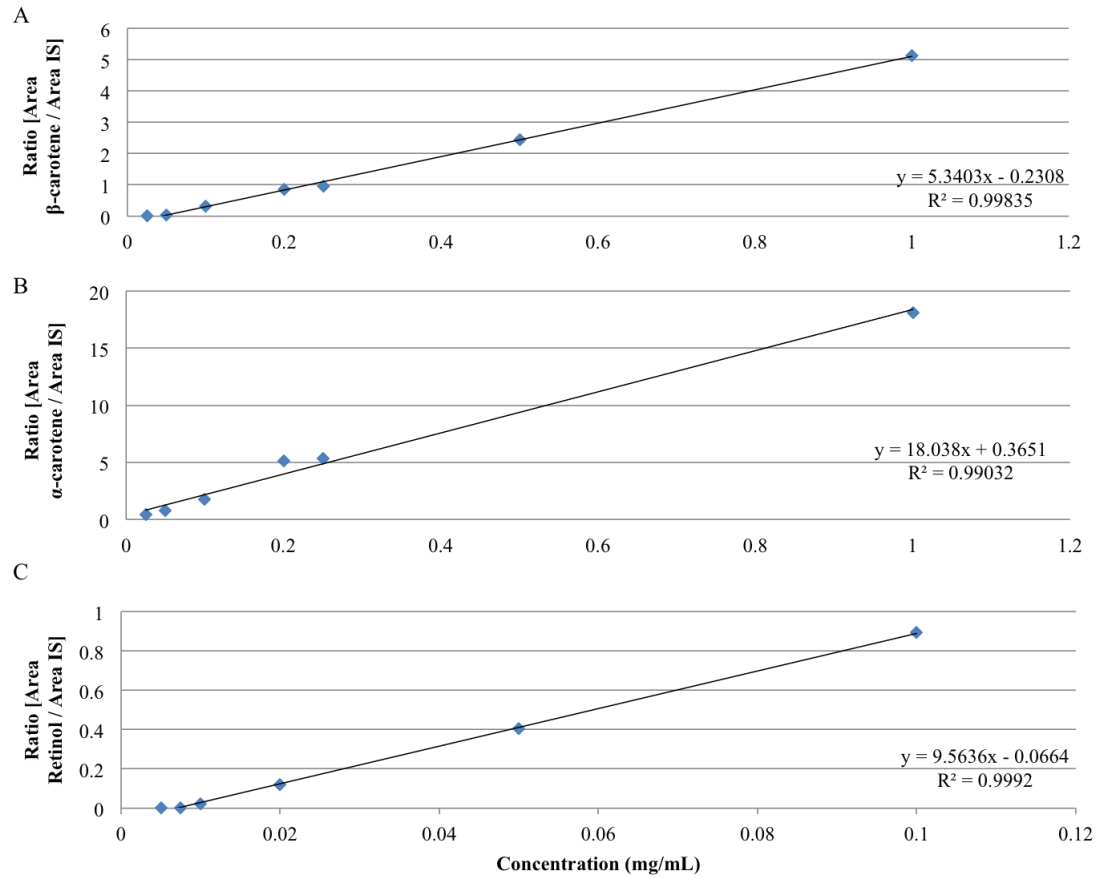**Liquid chromatography of retinoids and carotenoids**

Figure A-1: Sample calibration curves for β-carotene, α-carotene and retinol for plasma based on calibration standards. Concentrations expressed as the ratio between the compound area and the internal standard, retinyl acetate.

Table A-1: Linearity and calibration equations used for UPLC analysis.

Matrix	Compound	Equations	R Value
Plasma	α-carotene	y = 18.038x + 0.3651	R^2 = 0.9903
Plasma	β-carotene	y = 5.3403x − 0.2308	R^2 = 0.9983
Plasma	lutein	y = 22.924x − 0.145	R^2 = 0.9941
Plasma	zeaxanthin	y = 17.816x − 0.072	R^2 = 0.9984
Plasma	retinol	y = 9.5636x − 0.0664	R^2 = 0.9992
Diet	α-carotene	y = 14.795x + 1.123	R^2 = 0.9651
Diet	β-carotene	y = 4.424x - 0.006	R^2 = 0.9731
Diet	lutein	y = 21.821x - 0.0822	R^2 = 0.9975
Diet	zeaxanthin	y = 16.64x - 0.045	R^2 = 0.9964

Table A-2: Plasma retinoid and carotenoid percent changes from baseline to DURING and POST time points, in control and carrot groups.

| % change | Control (n=9) | | Carrot (n=10) | | Significant effects | | |
	DURING	POST	DURING	POST	Diet (P<)	Time (P<)	D*T (P<)
Retinol	-1.52(5.47)	12.03(6.12)	6.88(6.08)	16.69(6.18)	NS	0.005	NS
β-carotene	-13.29(4.30)	-7.31(3.90)	7.91(4.29)	8.57(7.17)	0.02	NS	0.02
Lutein	-4.03(3.88)	5.79(4.72)	-0.97(3.22)	0.06(5.82)	NS	NS	NS
Zeaxanthin	-3.27(2.93)	0.04(2.57)	0.72(3.65)	-1.21(5.25)	NS	NS	NS

Values expressed as mean(SEM). Significant effects of diet and time were identified with nested two-way ANOVA with repeated measures. NS, not significant.

Table A-3: Plasma lipids, glucose, and oxidative stress parameters percent changes from baseline to DURING and POST time points, in control and carrot groups.

	Control (n=9)		Carrot (n=9)		Significant effects		
% change	DURING	POST	DURING	POST	Diet (P<)	Time (P<)	D*T (P<)
Glucose	1.01(3.47)	3.57(5.94)	3.78(2.02)	9.28(2.07)	NS	0.05	NS
TC	-2.06(4.03)	6.67(2.94)	-0.15(5.07)	4.53(5.04)	NS	NS	NS
LDL	-2.88(13.17)	9.23(4.16)	-1.74(8.46)	-3.20(10.75)	NS	NS	NS
HDL	-3.92(4.25)	6.76(4.15)	4.74(5.36)	11.90(6.34)	NS	0.03	NS
VLDL	-6.18(11.03)	10.19(13.00)	-8.79(10.50)	11.78(14.36)	NS	NS	NS
TG	24.42(32.79)	10.27(13.62)	-9.32(10.15)	11.15(14.15)	NS	NS	NS
F2-Isoprostanes	N/A	0.40(3.65)	N/A	-4.96(3.06)	NS	NS	NS

Values expressed as mean(SEM). Significant effects of diet and time were identified with nested two-way ANOVA with repeated measures. HDL, high-density lipoprotein; LDL, low-density lipoprotein; NS, not significant; TC, total cholesterol; TG, triglyceride; VLDL, very-low density lipoprotein.

Enzyme-linked immunosorbent assay of 8-iso prostaglandin F2α in serum

The CusaBio (Cat# CSB-E12100h, Hubei, P.R. China) assay kit utilized a pre-coated antibody microplate with detection for 8-iso-PGF2α. The addition of a biotin-conjugated antibody, specific for 8-iso-PGF2α, produced biotinylated proteins. Once samples are incubated and washed, and horseradish peroxidase (HRP)-avidin was added in order to detect biotinylated antibodies. After the final wash to remove any unbound avidin-enzyme, a substrate solution, 3,3'5,5'-tetramethylbenzidine (TMB) was added to develop a color that is proportional to the amount of 8-iso-PGF2α bound in the initial step. The concentration of 8-iso-PGF2α is quantified through the intensity of the color measured at 450nm.

Chemicals, materials and preparation of standard stock solutions:

The kit supplied standard freeze-dried 8-iso-PGF2α (5000pg/mL), which was centrifuged at 6000rp for 30 seconds and reconstituted with 1 mL of sample diluent. Six tubes of calibrant were prepared using a 2-fold dilution series (2500, 1250, 625, 312, 156, 78, and 0 (blank) pg/mL). Following reconstitution of the 8-iso-PGF2α with 1mL of sample diluent, the standard was allowed to sit for 15 mins prior to use.

Reagents:

- Biotin-anitbody (1x) was reconstituted by diluting 10μL of biotin antibody concentrate (100x) with 990μL of biotin-antibody diluent.
- Detection reagent (HRP-avidin) (1x) was reconstituted by adding 10μL of HRP-avidin concentrate (100x) to 990μL of HRP-avidin diluent.
- Wash buffer (1x) was prepared by adding 480mL of distilled water to 20mL of Wash buffer Concentrate (25x).
- TMB substrate – amount needed.

- Stop solution – amount needed.

- Sample diluent – amount needed.

Sample preparation & procedure:

Aliquoted plasma from each participant was thawed on ice and no dilutions were required. Blank, standards, and samples were vortexted and 100µL of each were plated in duplicates on the same 96-well plate. Following plating, the plate was sealed and incubated for 2 hours at 37°C. After incubation, solution from each well was aspirated and discarded. 100µL of 1x biotin-antibody was added to each well, covered and incubated for 1 hour at 37°C. Each well was then aspirated using a multichannel pipette and washed with 200µL. Washing procedure was repeated 3 times. After the last wash, all remaining liquid was removed by inverting and blotting the plate against adsorbent paper. 100µL of 1x HRP-avidin was added to each dry well. The plate was sealed and incubated for 1 hour at 37°C.

HRP-avidin was aspirated from each well, and washing procedure was repeated 5 times. 90µL of TMB substrate was added to each dry well and the plate was protected from light and incubated for 15-30 mins at 37°C. 50µL of stop solution was added to each well and mixed by gently tapping the side of the plate to ensure thorough mixing. Absorbance of each well was read at 450nm using a Biotek Powerwave XS2 microplate reader (Biotek, Vermont, United States) with Gen5 software (Version 2.04, Biotek, Vermont, United States).

Enzyme-linked immunosorbent assay of malondialdehyde in plasma

The CusaBio (Cat# CSB-E08557h, Hubei, P.R. China) assay kit utilized a pre-coated antibody microplate with specific detection for malondialdehyde (MDA). Upon introduction of HRP-conjugated MDA, a competitive inhibition reaction occurs between

MDA and HRP-conjugated MDA with the pre-coated antibody specific for MDA. The more MDA present in the samples, the less antibody will be bound with HRP-conjugated MDA. Following the final wash, a substrate solution, 3,3'5,5'-tetramethylbenzidine (TMB) was added to develop a color that is inversely proportional to the amount of MDA present in the initial step. The concentration of MDA is quantified through the intensity of the color measured at 450nm.

Chemicals, materials and preparation of standard stock solutions:

The kit supplied pre-prepared MDA standard solutions (0.1, 0.4, 2, 10, 40 and 0 (blank) μg/mL).

Reagents:

- Wash buffer (1x) was reconstituted by diluting 15mL of wash buffer concentrate (20x) with 285mL of distilled water.
- HRP-conjugate – amount needed.
- Stop solution – amount needed.
- Substrate A and B – amount needed.

Sample preparation & procedure:

Aliquoted plasma from each participant was thawed on ice. No dilutions were required. Blank, standards, and samples were vortexed and 50μL of each were plated in triplicate and run on 2, 96-well plates. Following plating, 50μL of HRP-conjugate was added to each well, except the blank, mixed, and incubated for 1 hour at 37°C. After incubation, solution from each well was aspirated using a multichannel pipette, and washed with 200μL of 1x wash buffer. Washing procedure was repeated 3 times. After the last wash, any remaining liquid was removed by inverting, and blotting the plate against adsorbent paper. 50μL of both substrate A and B were added to each dry well and

incubated for 15 mins at 37°C. 50μL of stop solution was added to each well. Liquid was mixed by gently tapping the side of the plate. Absorbance of each well was read at 450nm using using a Biotek Powerwave XS2 microplate reader (Biotek, Vermont, United States) with Gen5 software (Version 2.04, Biotek, Vermont, United States). Absorbance was determined well within 10 mins of injecting the stop solution.
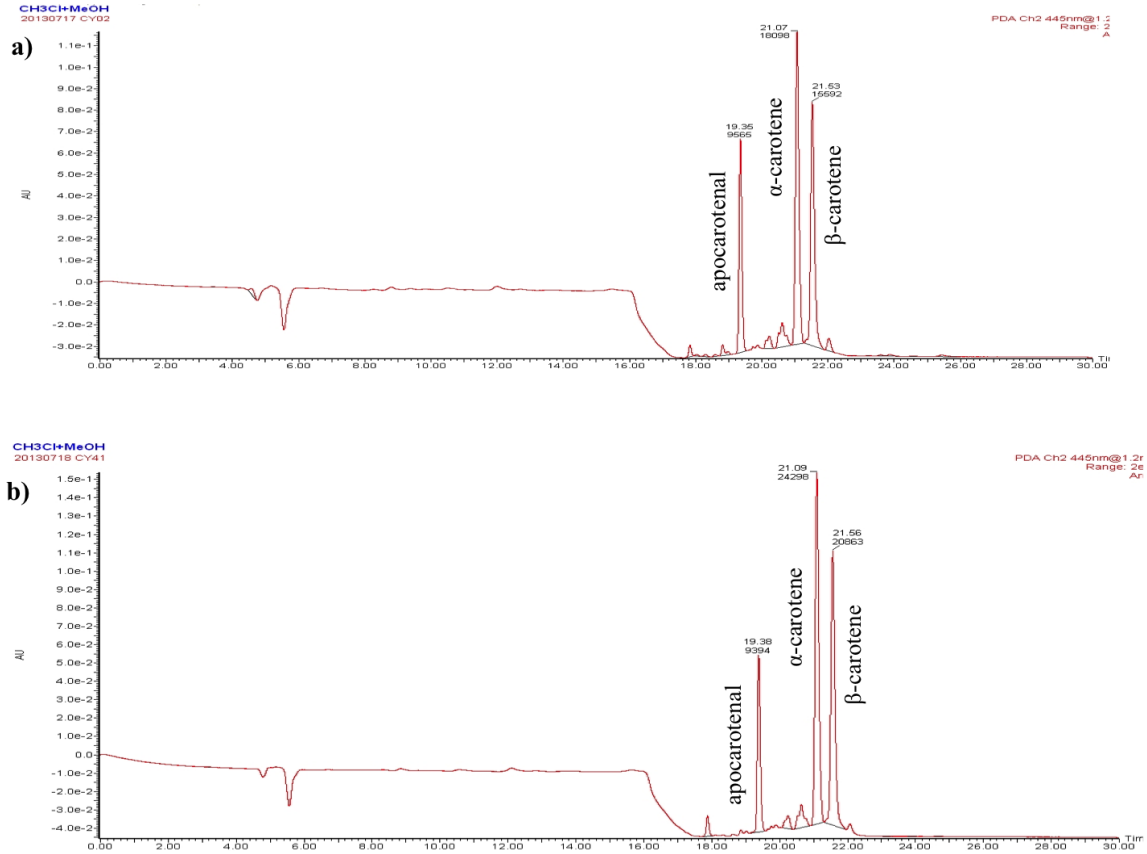
Figure B-1: Sample UPLC-PDA chromatogram of carrot powder diet. (a) Carrot powder enriched yogurt at week 0 upon receiving shipment, containing α-carotene, β-carotene, and internal standard apocarotenal measured at 445nm. (b) Carrot powder enriched yogurt at week 4 upon receiving shipment, containing α-carotene, β-carotene, and internal standard apocarotenal measured at 445nm.
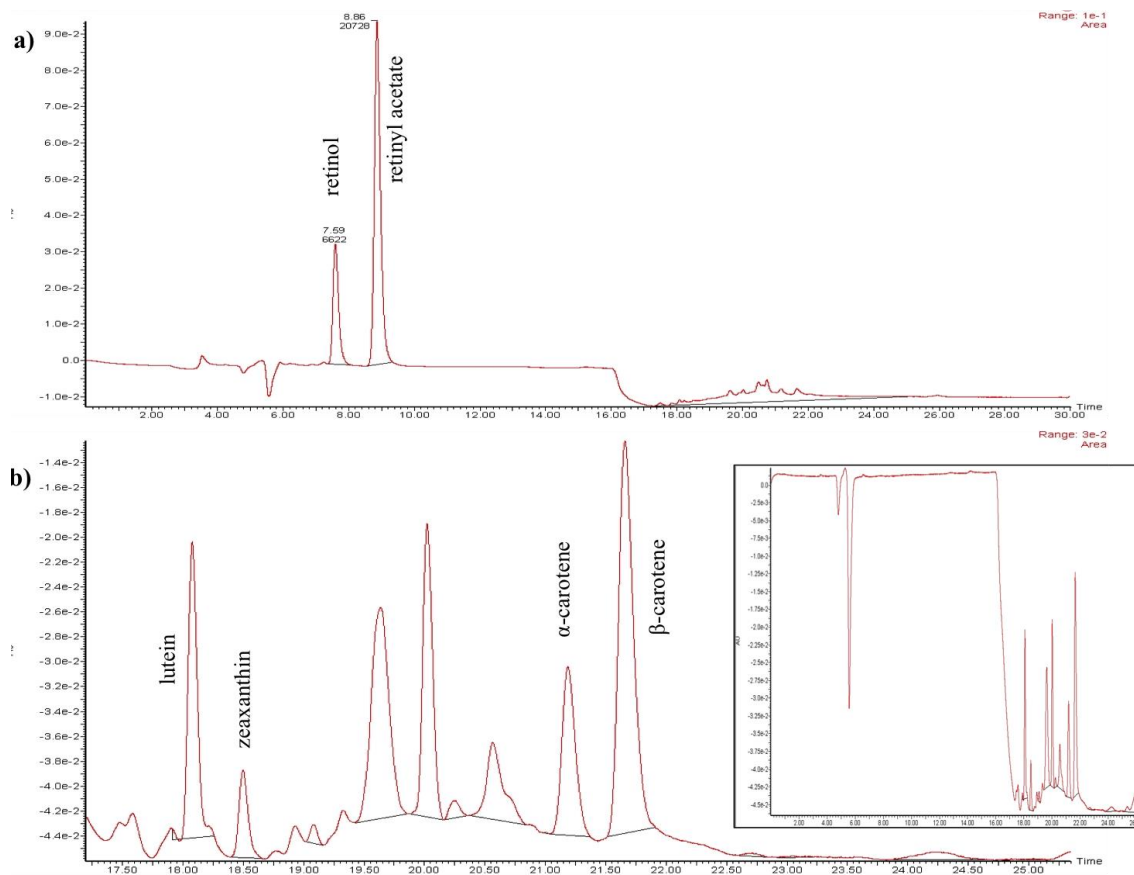
Figure B-2: Sample UPLC-PDA plasma carotenoid and retinoid chromatograms at week 0. (a) Sample retinoid chromatogram from a participant in the carrot group containing retinol and internal standard, retinyl acetate at 325nm. (b) Enhanced view of a sample carotenoid chromatogram at 17.5-25 mins from a participant in the carrot group containing, lutein, zeaxanthin, α-carotene, and β-carotene with identification set at 445nm.
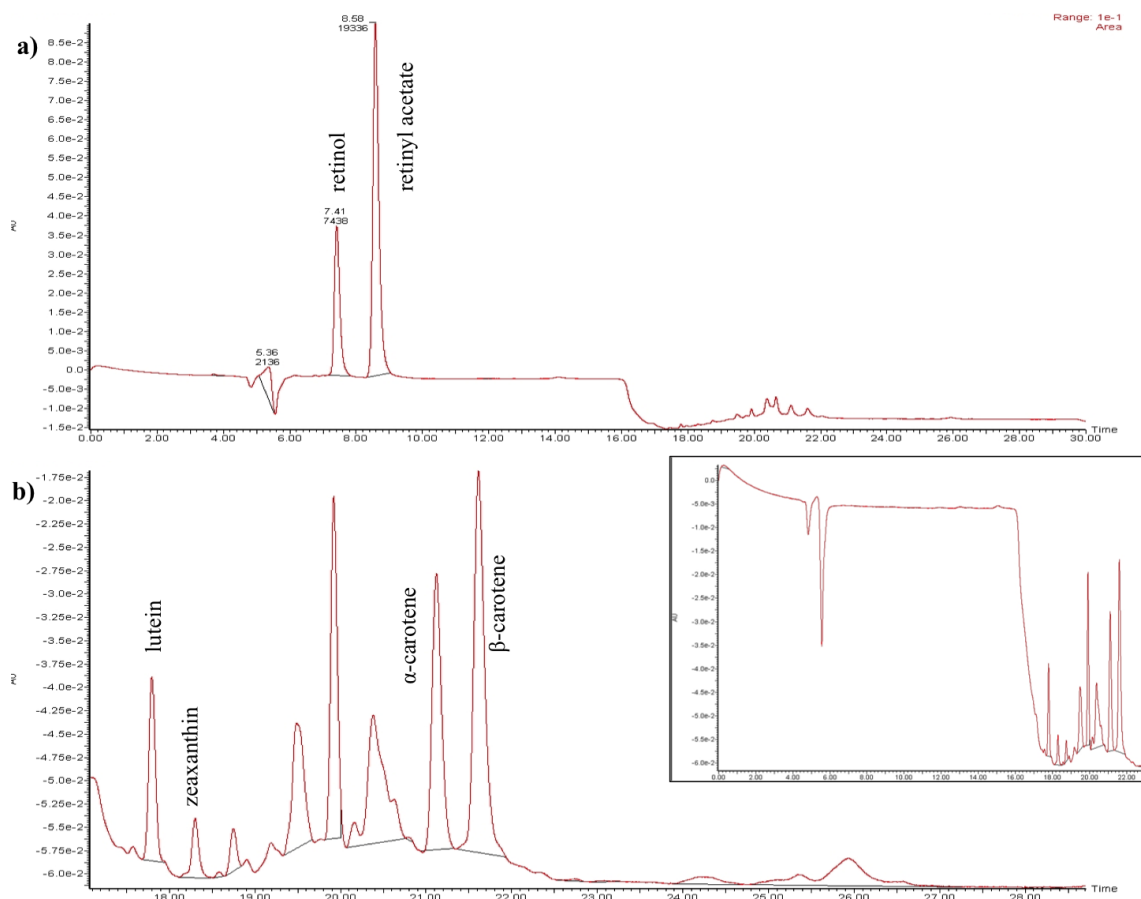
Figure B-3: Sample UPLC-PDA plasma carotenoid and retinoid chromatograms at week 2. (a) Sample retinoid chromatogram from a participant in the carrot group containing retinol and internal standard, retinyl acetate at 325nm. (b) Enhanced view of a sample carotenoid chromatogram at 17.5-28 mins from a participant in the carrot group containing, lutein, zeaxanthin, α-carotene, and β-carotene with identification set at 445nm.
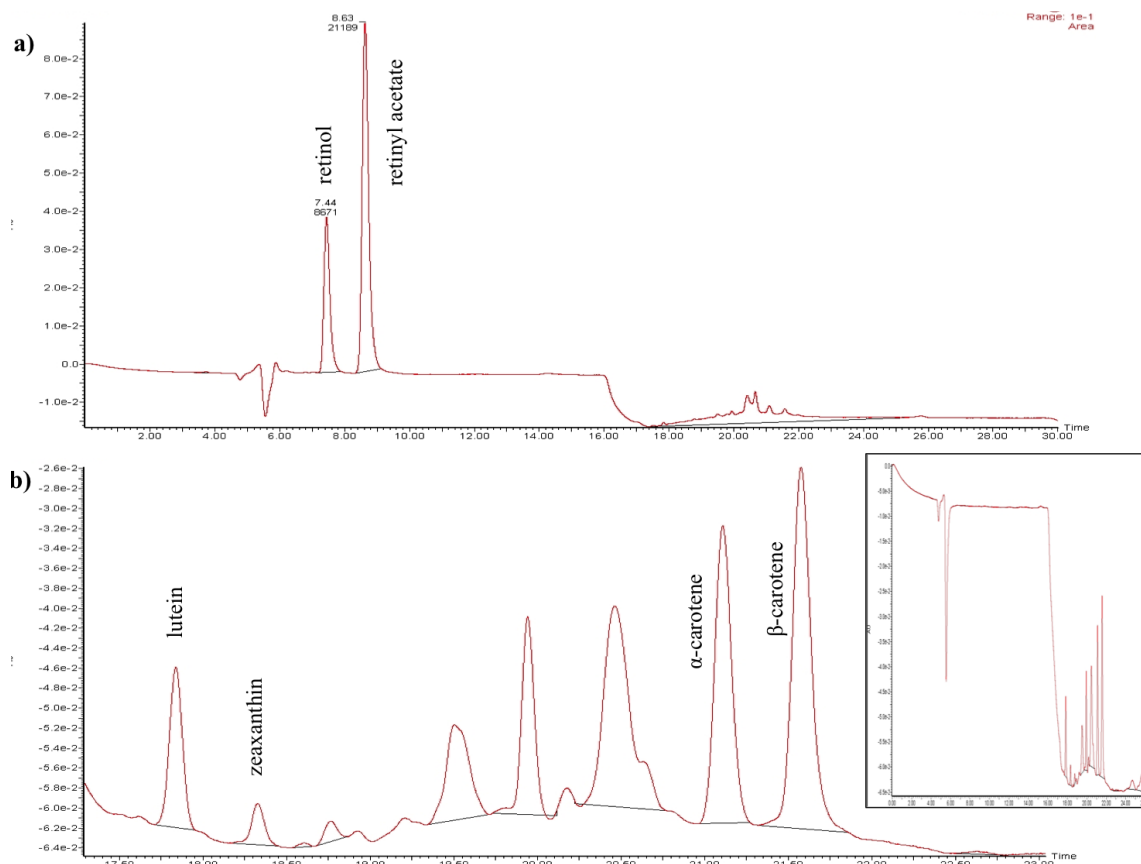
Figure B-4: Sample UPLC-PDA plasma carotenoid and retinoid chromatograms at week 4. (a) Sample retinoid chromatogram from a participant in the carrot group containing retinol and internal standard, retinyl acetate at 325nm. (b) Enhanced view of a sample carotenoid chromatogram at 17.5-23 mins from a participant in the carrot group containing, lutein, zeaxanthin, α-carotene, and β-carotene with identification set at 445nm.