THE EFFECTS OF CARROT CAROTENOIDS ON DIABETIC RETINOPATHY IN TYPE 1 DIABETES MELLITUS

by

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Abstract

While carotenoids are essential for visual function, their potential role in diabetic retinopathy is not known. By providing carrot powder, this study examined carotenoid metabolism and visual function in Type 1 diabetes. Wistar rats (n=30) were assigned to diet either with or without carrot enrichment (15%, w/w) for 12 weeks. Type 1 diabetes was induced with streptozotocin at 3 weeks. Retinal function and anatomical integrity were assessed along with retinoid and carotenoid levels in the serum, liver, and retina. Loss of ERG oscillatory potentials, with normal histology indicated early stage retinopathy. Healthy animals fed carrot diet showed highest b-wave amplitudes; reflecting higher phototransduction. Diabetic animals fed carrot diet had the lowest b-wave amplitudes, reduced retinoids liver reserves, and highest α- and β-carotene, suggesting disturbance of conversion during diabetes. Consequently carrot powder at concentrations used by this study cannot be recommended for diabetic retinopathy.

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List of Abbreviations

8-OHdG – oxidatively modified DNA 8-hydroxy-2" deoxyguanosine

A2E – N-retinyl-N-retinylidene ethanolamine

ABCA1 – ATP-binding cassette transporter ABCA1

AF – skin autofluorescence

AGE – advanced glycation end product

AI – adequate intake

ALE – advanced lipidoxidation end product

ALT – alanine aminotransferase

ARAT – acyl-CoA-retinol acyltransferase

ATBC – Alpha-Tocopherol, Beta-Carotene Cancer Prevention Trial

BBdp – biobreeding diabetic prone rats

BCDO2 – β-carotene dioxygenase 2

BCMO1 – β-carotene 15,15'-monooxygenase

CARET – Carotene and Retinol Efficacy Trial

CEP – carboxyethylpyrrole

cGMP – photoreceptor-specific G protein

CML – N-$^{\varepsilon}$(carboxymethyl) lysine

CRALBP – cellular retinaldehyde-binding protein

CRP – C-reactive protein

DAPI – 4'-6-Diamidino-2-phenylindole stain

DHA – docosahexaenoic acid C22:6n-3

EAR – estimated average requirement

ELISA – enzyme-linked immunosorbent assay

ERG – full-field electroretinogram

GFAP – glial fibrillary acidic protein

GLUT-1 – glucose transporter 1

HDL – high density lipoprotein

INL – inner nuclear layer

IRBP – Inter-photoreceptor retinoid binding protein

IU – international units

LDL – low density lipoprotein

LPL – lipoprotein lipase

LRAT – lecithin-retinol acyltransferase

MnSOD – manganese superoxide dismutase

MPOD – macular pigment optical density

ONL – outer nuclear layer

PDR – proliferative diabetic retinopathy

RAE – retinol activity equivalents

RAGE – receptor for advanced glycation end products

RBP(4) – retinol binding protein (4)

RDA – recommended dietary allowance

RE – retinyl ester

REH – retinyl ester hydrolase

RPE65 – retinal pigment epithelium-specific 65 kDa protein

ROS – reactive oxygen species

SR-BI – scavenger receptor class B member 1

STRA6 – stimulated by the retinoic acid 6 gene

STZ – streptozotocin

TTR – transthyretin

UPLC-PDA –ultra performance liquid chromatography with photodiode array detector

WHO – World Health Organization

Chapter 1: INTRODUCTION

Diabetic retinopathy is a major long-term complication of the disease, having a significant impact on life quality of the individual. The vast majority (99%) of individuals with Type 1 diabetes will develop some degree of retinopathy over the course of their lives, as will greater than 60% of individuals with Type 2 diabetes. Breakdown of the blood-retinal barrier, basement membrane thickening, lower levels of nitric oxide sythetase and vasoregulatory abnormalities, all contribute to the pathophysiology of diabetic retinopathy (Stitt et al., 2000). Duration of disease is key factor in predicting rate of retinopathy, but is not the sole determinant of progression. Sixty % will develop proliferative retinopathy in 15 to 20 years (Jawa et al., 2004). Blindness in adults as a result of diabetic retinopathy affects 23% of all Type 1 and 14% of Type 2 diabetic individuals and is the leading cause of blindness in developed countries (Congdon et al., 2003). Although smoking cessation, good glycemic and blood pressure control and treatment of dyslipidemia, contribute to a reduction in risk, unfortunately retinopathy still develops in 12% of patients who follow these recommendations (Chaturvedi et al., 2001).

Carotenoids are well known as antioxidants and some are precursors of Vitamin A, an important component of visual cycle in the retina. However, understanding of carotenoid metabolism and function in diabetic retinopathy is incomplete. Lack of knowledge has hindered recommendations for carotenoid intake in health and disease complications including diabetic retinopathy. Evidently, it is necessary to investigate new therapeutic strategies to prevent or stop this detrimental disease.

The following chapter provides a basic overview of carotenoid and vitamin A metabolism in health, diabetes and with existing dietary recommendations.

Carotenoid & Vitamin A Metabolism

Classifications

Of the approximately 600 carotenoids isolated from natural sources, only a select group, β-carotene (Male:0.47 μmol/L Female: 0.41 μmol/L), α-carotene (Male:0.065 μmol/L Female:0.081 μmol/L), β-cryptoxanthin (0.13 μmol/L), lycopene (Male:0.47 μmol/L Female:0.41 μmol/L), lutein and zeaxanthin (0.35 μmol/L), are found in significantly in serum of humans (Olson et al., 1999; Kurlandsky et al. 1995).

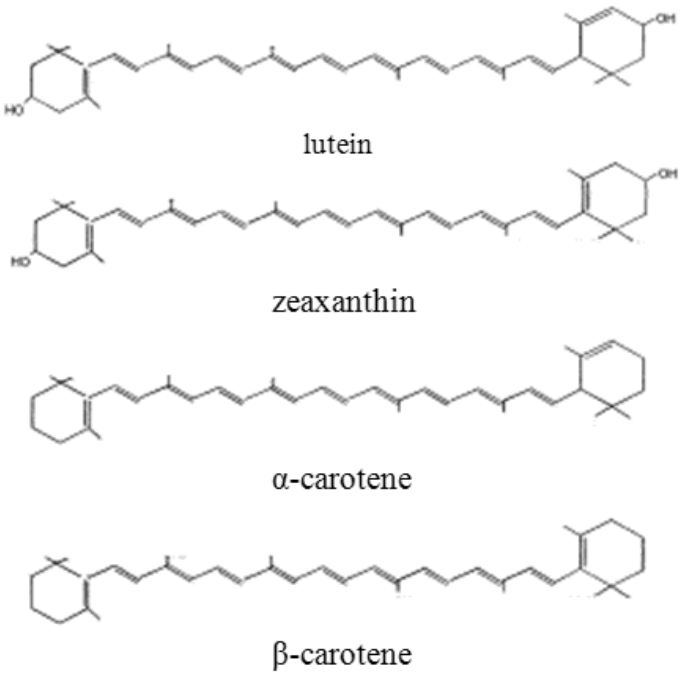

Figure 1-1: Chemical structures of major carotenoids found in carrots which appear in human and animal systems.

Vitamin A can either be obtained from preformed vitamin A, such as retinol and retinyl esters, or from provitamin A carotenoids β-carotene, α-carotene, and β-cryptoxanthin which are converted to vitamin A in the body (Fig. 1-1 & 1-2).

Absorption & metabolism

Vitamin A is the precursor for at least two essential biologically active molecules; 11-*cis*-retinal, a required substrate for chromophore development in the visual cycle (refer to page 21) and all-*trans*-retinoic acid, regulator of retinoid homeostasis and hormone-like ligand of nuclear receptors. Dietary preformed vitamin A in the retinyl ester form is obtained from animal sources or as provitamin A carotenoids, obtained from dark leafy green and orange vegetables and fruits.

On consumption, retinoids and retinyl esters are emulsified, entering the enterocyte by diffusion whereby a series of enzymatic steps including retinol cellular retinol-binding protein I&II (CRBP I & II), basolateral transporter ABCA1, lecithin-retinol acyltransferase and acyl-CoA-retinol acyltransferase incorporate retinyl esters (REs), primarily comprised of retinyl palmitate, into chylomicrons or as free retinol for distribution into the lymphatic system and circulation (During and Harrison, 2007; Batten et al., 2004; MacDonald and Ong, 1988). In contrast to retinoid processing, absorption of carotenoids from the intestinal lumen into the enterocyte does not occur passively. Recent findings in Caco-2 human colonic culture models depict a highly regulated system of transporters and receptors (SR-BI and BCMO1) that are highly saturable and respectively absorb and convert β-carotene to retinol (Lobo et al., 2010).

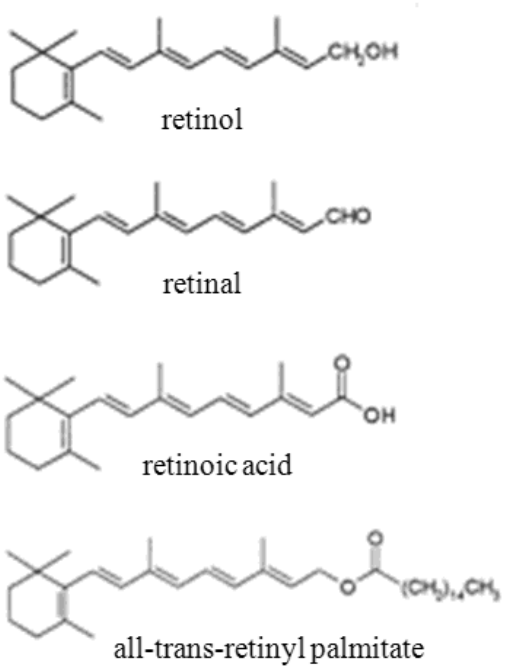

Figure 1-2: Chemical structures of key vitamin A derivatives found in human and animal systems.

In the enterocytes of the intestinal mucosa, three mechanisms of carotenoid to retinoid conversion shunt vitamin A isomers into the circulatory system (Fig. 1-3). The major route, β-carotene 15,15'-monooxygenase (BCMO1), cleaves β-carotene symmetrically at the central double bond to yield two molecules of retinal (von Lintig and Vogt, 2000; Voshol et al., 2001; Cai et al., 2001; Redmond et al., 2001). A second route allows for absorption of intact carotenoids which accounts for 40% of β-carotene consumed by humans (Castenmiller and West, 1998). The third recently discovered route, is asymmetric cleavage of β-carotene by β-carotene -9′,10′-dioxygenase which results in the creation of β-apo-carotenoic acids which can then be shortened to retinoic acid (Kiefer et al., 2001).

Studies have shown controversial results on the competition of simultaneous ingestion of carotenoids. β-carotene has shown inhibition of lutein and canthaxanthin absorption (Maiani et al., 2009) or no competition with β-cryptoxanthin, α-carotene, lutein

and zeaxanthin (Hoppe et al., 2003). Resultantly, there is a need for additional investigation into the absorption and integration of mixed carotenoid formulations and their impact on tissue composition and metabolic processes. Little research exists on the utilization of carotenoids as found in naturally occurring food matrices, such as that of the carrot. In the retinyl ester or retinol form, substrate travels through the portal venous system for uptake into target tissues (Wang and Krinsky, 1998). In fasting vertebrates, 95% of the vitamin A in serum is found in the unesterified form, retinol, regardless of intake. In the case of retinyl esters, serum lipoprotein lipase catalyzes the release of glycerol, fatty acids, and retinol from within the circulating chylomicrons. All components can then be absorbed by peripheral tissues.

The liver is the primary storage site, and regulator of vitamin A homeostasis, storing 50-85% of the body's reserves in vitamin A (0.147-0.441 umol/g) as REs (Blaner et al., 2009; Goodman, 1984; Olson, 1984; Penniston and Tanumihardjo, 2006). For release into the circulation REs must be unesterified into retinol by retinol ester hydrolase (REH). Unesterified all-*trans*-retinol binds to retinol binding protein (RBP) and transthyretin (TTR) which are synthesized in the liver. Expression of REH is tightly regulated by liver vitamin A status. Under normal conditions, the body maintains serum retinol levels at 1-3μmol/L, which vary little due to homeostatic regulation, even in situations of deficiency (Olson et al., 1999).

Circulating REs, α-retinol and carotenoids in chylomicrons are taken up by target tissues that include the colon, exocrine glands in pancreas, glandular cells in prostate, mammary tissue, endometrial, kidney tubular cells, testis, ovary, adrenal gland, skeletal muscle cells and keratinocytes of skin squamous epithelium, but not as readily in the retina (Lindqvist et al., 2005), which will be discussed in greater detail in the following chapter.

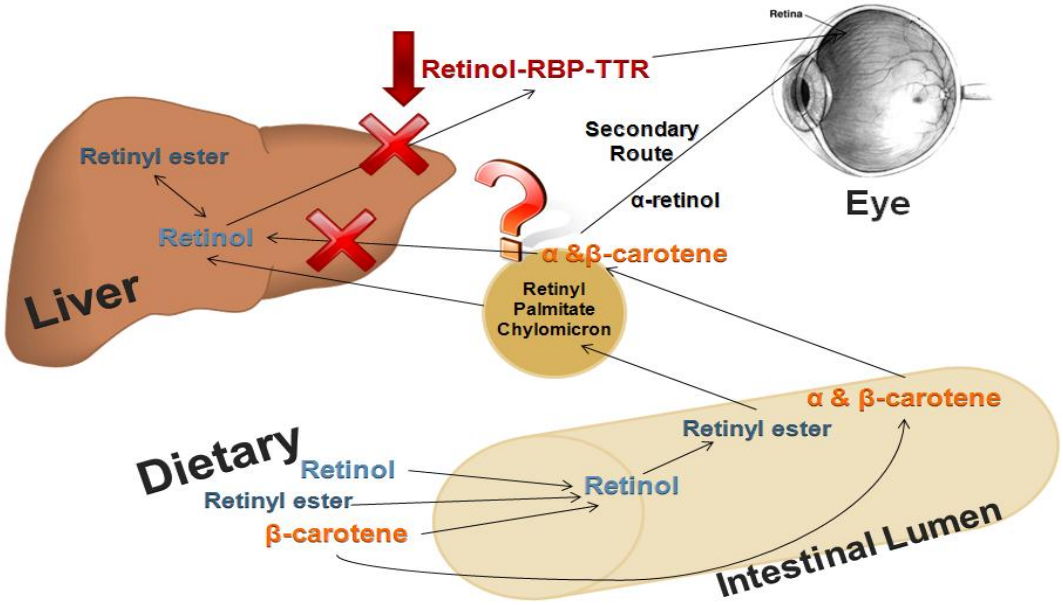

Figure 1-3: Basic vitamin A and carotenoid transportation and conversion in the body. Alterations in metabolism reported in Type 1 diabetes denoted with X and question mark.

The kidneys are the primary site of retinoid excretion and retinoid binding protein recycling. In isotopic studies, a majority of the carotene isotope was excreted in less than 48 hours through the feces (20-60%), urine (10-30%), breath (10%), saliva, sweat and tears (unquantified) (Dueker et al., 2000; Sauberlich et al., 1974; Wiggert and Chader, 1985). Plasma holo-RBP complex is partially filtered in the glomerulus when TTR is bound to RBP, which in turn is capable of binding to megalin (gp330) in the renal proximal tubule cells for recycling in a healthy state (Christensen et al., 1999). Meanwhile, unbound RBP is excreted by the renal proximal tubule cells.

Vitamin A deficiency and toxicity

The World Health Organization (WHO) indicates that vitamin A deficiency still remains a significant public health problem at the global level. Vitamin A deficiency is the lead cause of preventable childhood blindness (The World Health Organization, 2009). Children who are vitamin A deficient are also 23% more likely to die from measles, malaria or diarrhea (Calloway et al., 1993). WHO estimates that 33% of pre-school aged

children and 15% of pregnant women do not have enough vitamin A in their daily diet. Furthermore, another 5.2 million pre-school-age children suffer from the clinical symptoms of deficiency worldwide.

Suboptimal vitamin A intake has also been observed in developed countries. According to the 2004 Canadian Community Health Survey, more than 35% of Canadians age 19 and over consumed vitamin A in quantities below the estimated average requirement (EAR), which meets the requirements of 50% of the population. The prevalence of inadequate intakes was greater than 40% in most adult age categories (Health Canada, 2009a). In another recent large survey of 18 Canadian Inuit communities based on 24-hour recalls and 7-day food records, vitamin A inadequacy was found to be of significant concern for young adults. Based on EAR for retinol activity equivalents, 68% of men and 60% of women (15–40 years of age) had an intake below the EAR (Egeland et al., 2004). This data is particularly concerning for women of childbearing age, as stress of pregnancy can further deplete vitamin A reserves, leading to night blindness and more adverse signs and symptoms of deficiency.

Vitamin A deficiency can occur as either a primary (dietary) or secondary deficiency. Secondary deficiency, although less common, is associated with chronic malabsorption of lipids, impaired bile production and chronic exposure to oxidants which can result in serious physiological alterations to visual function. Borderline-low plasma concentrations of retinol have been exhibited in Type 1 diabetes mellitus (Basu et al., 1989; Lu et al., 2000a; Tuitoek et al., 1996c) suggesting a possible connection between diabetic retinopathy and blindness relative to poor vitamin A metabolism and distribution.

Vitamin A deficiency has many severe health consequences if left untreated. Pregnant women, infants and children appear to face the greatest risk. Visual disturbances are one of the most debilitating symptoms of vitamin A deficiency. Under declining levels

of vitamin A, night blindness from a depletion of 11-*cis*-retinal provides little substrate for rhodopsin and subsequent photooxidation in the visual cycle of rod cells. Xerophthalmia and other ocular changes include keratinizing metaplasia of the conjunctiva (Bitot's spots) caused when insufficient vitamin A is available to promote secretion of mucus in the epithelium cells of the eye. In turn, the collection of dead cells and bacterial growth cause infection and opaque plaques destroying the surface of the cornea causing blindness.

In murine vitamin A deficiency models, RE synthesis for storage is decreased and hepatocytes rapidly repackage retinol for secretion and distribution into plasma bound to RBP (Dixon and Goodman, 1987). Concurrently, an increase in RBP and TTR is expressed by liver hepatocytes to act as carriers of retinol for distribution in the body where it is most needed. In extreme vitamin A deficiency, TTR plasma levels remain constant meanwhile RBP levels are decreased (Dixon and Goodman, 1987; Rosales et al., 1996). Conversely, in states of infection, inflammation and the acute phase response, synthesis of both TTR and RBP are found to be decreased, suggesting that inflammation itself creates a state of temporary vitamin A deficiency (Rosales et al., 1996).

In a normal healthy state, vitamin A derivative retinoic acid acts to modulate insulin production and secretion and participates in the modulation of genes in islets of Langerhans of the pancreas (Berdanier et al., 2001). Studies on vitamin A deficiency in otherwise healthy animals have demonstrated impaired fetal islet development and subsequent defective insulin release in response to serum glucose levels (Berdanier et al., 2001; Matthews et al., 2004) indicating a close relationship between vitamin A and glucose control early in life. Insulin release and glucose intolerance improved with dietary vitamin A repletion suggesting a critical relationship between the two (Chertow et al., 1987).

In 2008 it was estimated that a global annual investment of US$60 million in vitamin A and zinc supplementation for children with deficiency would yield benefits of more than US$1 billion per year to the global health system (Copenhagen Consensus, 2008). Considerable effort has also been made to increase dietary carotenoid intake to combat vitamin A deficiency. Food-based approaches, such as agriculture of β-carotene rich vegetables, have been considered as an appropriate and sustainable complement to supplementation programs in the prevention of vitamin A deficiency (Low et al., 2007) . However, the most common method remains delivery of high-dose oral vitamin A supplements in high risk counties. Semi-annual high dose retinyl palmitate therapy for deficiency ranges from 50,000-200,000 IU and is contingent on age and severity of deficiency and is highly absorbed (World Health Organization, 1976). Toxicity is not seen in this population, nor in most supplemental trials as it requires prolonged daily dietary intakes greater than 25,000IU for more than 6 years or greater than 100,000 IU for more than 6 months. It is commonly a result of excessive supplementation and food faddism (Penniston and Tanumihardjo, 2006). Side effects of toxicity are dose dependent, with effects ranging from skin irritations and headache, to teratogenicity and bone fracture in the most severe cases. Consequently, vitamin A therapy is effective, but not without the risk of serious toxicity without careful monitoring.

Dietary recommendations

Health Canada's recommended dietary allowance for vitamin A is 900µg/daily for men and 700µg/daily for women from dietary sources. Based on this recommendation, a daily β-carotene intake ranging from 130µg - 6000µg has also been devised (Health Canada, 2010). Recommendations also exist for pregnant women and individuals at various stages in the lifecycle.

A system of retinol equivalency has been established to determine the vitamin A (retinol) conversion rate of provitamin A carotenoids. Current research supports 1 retinol activity equivalent (RAE) as being the equivalent to 1 μg of retinol, 12 μg of β -carotene or 24 μg of other pro-vitamin A carotenoids (α-carotene and β-cryptoxanthin) (Health Canada, 2010).

The upper tolerable limit set for preformed vitamin A is 3000 μg, or retinol equivalents (RAE)/d. However, in addressing the possible risk of bone fracture and osteoporosis in postmenopausal women, restricting intake to 1500 μg RAE/d has been recommended, but further evidence is needed to substantiate this recommendation or any guidelines for β-carotene supplementation (Vivat-Hannah and Zusi, 2005).

Carotenoids & vitamin A in visual function

Retina

The retina is a light-sensitive tissue lining the inner surface of the eye made up of complex layers of neurons interconnected by synapses. Light striking the photoreceptor cells of the retina triggers a cascade of electrical and chemical events sent to a variety of visual centers in the brain. Photoreceptors exist as two cell types; rods which provide contrast and functions mainly in dim light and cones which perceive colours and are required for bright light vision.

Vitamin A and provitamin A carotenoid metabolism in the visual cycle

The retinas of chordates accept all-*trans*-retinol, and vitamin A precursor β-carotene, but not retinyl esters (Bhatti et al., 2003). Little is understood about β-carotene, α-carotene, and utilization of their metabolites by the retina. Defects in nearly every conversion enzyme of vitamin A metabolism in the visual cycle cause inherited retinal dystrophies in humans and animals either through impaired synthesis of visual

chromophores, or cytotoxic accumulation of chromophore and all-trans-retinal in the photoreceptors (von Lintig et al., 2010).

In order for any vitamin A to be integrated into phototransduction and the visual cycle, the major route is by liver and the peripheral body stores re-secreting holo-RBP4 into the circulation (Blomhoff et al., 1990). Substrate for the visual cycle enters into the retinal pigment epithelium lumen by the retinoic acid 6 gene (STRA6), and scavenger receptor class B member 1 (SR-BI) (During et al., 2008; Isken et al., 2008). Carotenoid enzymes β-carotene 15,15'-monooxygenase (BCMO1), and to lesser degree β-carotene dioxygenase 2 (BCDO2) are also expressed at the retinal pigment epithelium and ciliary body suggesting that β-carotene contributes to the retinal content of the eye, although mechanisms have yet to be clearly elucidated (Chichili et al., 2005; Lindqvist et al., 2005).

Once in the retinal pigment epithelium, all-*trans*-retinol is stored as stable, readily-accessible, hydrophobic droplet-like retinosomes of all-*trans*-retinyl esters (Imanishi et al., 2004). Excess all-*trans*-retinyl esters are catalyzed to 11-*cis*-retinol by RPE65 in the lipid bilayer and transported for binding to opsin in the photoreceptors (Redmond et al., 2005; Moiseyev et al., 2006). In genetic mutations with low RPE65, retinyl ester accumulation leads to chromophore-deficiency further along the visual cycle pathway (Samardzija et al., 2008).

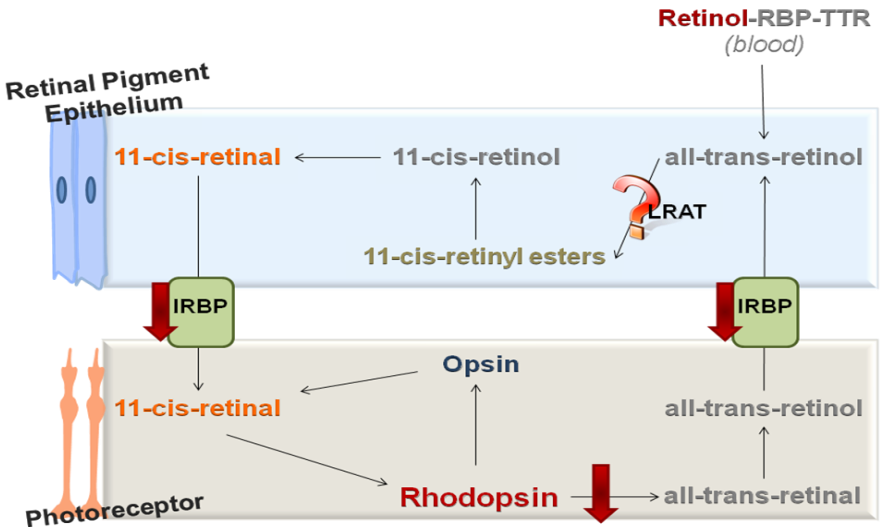

Figure 1-4: Vitamin A conversion in the visual cycle. Red arrows and question mark depict suspected dysregulations in diabetes.

After phototransduction, all-*trans*-retinol is oxidized by a series of retinol dehydrogenases (RDH5, RDH10 and RDH11) to form the substrate 11-*cis*-retinal while being bound to cellular retinaldehyde-binding protein (CRALBP) (von Lintig et al., 2010). CRALBP then mediates 11-*cis*-retinal transport to the serum membrane surface of the RPE where it is then passed off to inter-photoreceptor retinoid binding protein (IRBP), which contains 3-4 retinoid-binding sites per molecule (Fig. 4). IRBP and 11-*cis*-retinal enter the photoreceptor rod outer segment where 11-*cis*-retinal binds by Schiff-base linkage to a membrane-embedded Lysine residue in opsin, forming the essential visual pigment rhodopsin (Filipek et al., 2003).

Light entering the retina triggers an 11-*cis* to all-*trans* isomerization of the chromophore, activating rhodopsin and catalyzing a signal cascade involving photoreceptor-specific G protein transducin and cGMP that results in plasma membrane hyperpolarization. Rhodopsin splits into opsin and the all-*trans*-retinal. For sustained vision, the all-*trans*-retinal must be continuously recycled by enzymatic RDH8 and RDH12 reduction to all-*trans*-retinol with cofactor NADPH (Maeda et al., 2007). All-

trans-retinol is then shunted to the retinal pigment epithelium by IRBP and CRBP1, where it can be stored or recycled as visual chromophore.

Overall, cone photoreceptors are outnumbered more than 20:1 by rod cells in the human eye, and are even fewer in nocturnal animals (Mustafi et al., 2009). However, cone cells which mediate daylight vision, and are concentrated in the fovea, the primary center for mediating fine detail vision, are also critical for visual acuity and color discrimination. A cone-specific regeneration pathway to prevent cone/rod competition for 11-*cis*-retinal requires mechanistic confirmation. It is hypothesized that in the cone-specific visual cycle, all-*trans*-retinol is taken up by Müller cells where it is directly isomerized to the 11-*cis* configuration and esterified to 11-*cis*-retinyl esters by acyl-CoA: retinol acyltransferase (ARAT) (Mata et al., 2002; Mata et al., 2005). Reserves can then be mobilized by 11-*cis*-retinyl ester hydrolase (REH) to transport 11-*cis*-retinol to cone cells (Mata et al., 2002). Ultimate confirmation of this pathway still requires additional evidence and identification of the respective genes.

Other carotenoids in visual function

Epidemiological studies suggest subjects with high dietary intakes or high serum concentrations of lutein and zeaxanthin have a reduced risk of age-related macular degeneration (AMD) and cataracts (Gale et al., 2003; Snellen et al., 2002; Eye Disease Case-Control Study Group; 1993). For decades, non-provitamin A carotenoid xanthophylls, lutein and zeaxanthin have been known to exist in high concentrations in the fovea, the region of high density cones, although their functions have yet to be fully elucidated. Existing research highlights the ability of carotenoids to acts as antioxidants and absorbers of reactive oxygen species (ROS) and phototoxic light under normal conditions (Bendich and Olson, 1989; Krinsky, 1989; Flather, Lonn, Farkouh, & Yusuf, 1995; Jha et al. 1995). Provided that the retina is the only neural tissue which is subjected

to direct, constant exposure to light, it consumes more oxygen for its size than any other part of the human body, and is associated with the generation of elevated amounts of ROS. Many clinical trials have assessed the benefits of lutein and zeaxanthin supplementation on retinal function and oxidative protection in AMD. Some have noted a increases in lutein and zeaxanthin, as assessed with optical imaging in the fovea, was associated with visual loss prevention (Barker, 2010). No human or animal studies to date have uniquely assessed the impact of carotenoid supplementation in the diabetic retina, characterized by high levels of oxidative stress. This presents a novel opportunity for further research to determine the effects of a whole food containing carotenoids on visual function in diabetic retinopathy.

Vitamin A in Type 1 diabetes

Diabetes mellitus Type 1, otherwise known as insulin-dependent diabetes mellitus, is a form of diabetes that may result from autoimmune destruction of insulin-producing β-cells from the islets of Langerhans of the pancreas. Causes are still not fully understood. Long-term complications include cardiovascular disease, cerebrovascular accidents, and peripheral vascular disease (macrovascular) and nephropathy, neuropathy and retinopathy (microvascular).

It is estimated that annually some 76,000 children aged under 15 years develop Type 1 diabetes worldwide (International Diabetes Federation, 2010). Of the estimated 480,000 children with Type 1 diabetes, 24% come from the South-East Asian Region, but the European Region, where the most reliable and up-to-date estimates of incidence are available, come a close second (23%). The prevalence of Type 1 diabetes continues to rise globally. Timely studies of therapeutic strategies to better manage macrovascular and microvascular complications are needed to improve quality of life.

A variety of biochemical changes occur in the progression of diabetes and diabetic retinopathy with cascading, systemic effects. Among the hallmarks, the lack of insulin increases blood and urine glucose levels which are eventually fatal if not treated with insulin administration. Diabetes is confirmed in patients by the elevated serum glucose ($\geq$ 11.1 mmol/L at any time, $\geq$ 7.0 mmol/L fasting) and glycosylated hemoglobin (HbA1c $\geq$ 6.5%) (International Diabetes Federation, 2010). Type 1 diabetes is also characterized by altered metabolism and nutritional status of a variety of vitamins and minerals, including vitamin A. The existing literature demonstrates an association between Type 1 diabetes and altered vitamin A status in both animals and humans.

1. Vitamin A mobilization and transport

Altered serum concentrations of all-*trans*-retinol and its carrier proteins, RBP4 and TTR have been demonstrated in both Type 1 diabetic animals and humans, independent of dietary intake. This does not appear to be the case for individuals living with Type 2 diabetes, who may present with normal concentrations (Abahusain et al., 1999). Many studies of Type 1 diabetes mellitus demonstrate low-normal serum and hepatic concentrations of retinol and its carriers in adults (Basu et al., 1989; Lu et al., 2000a; Tuitoek et al., 1996b). One human study of adult identical twin pairs discordant for Type 1 observed the average mean serum retinol of Type 1 diabetics to be 46µg/dl, whereby their healthy matched pairs had levels of 60.9µg/dl (Dubrey et al., 1997). Concurrently they found levels of RBP reduced to 6.2mg/dl compared with 7.6mg/dl in the healthy controls. Results were not significant between unrelated twins, but were consistent between families, suggesting some level of inheritability for RBP transporter concentration. The decrease in retinol, RBP4 and TTR highlighted by the study is also consistent with states of systemic inflammation, infection, and acute phase response, and is measured by C-reactive protein (CRP) (Espe et al., 2007). In nutritional intervention, elevated CRP has been used to identify false vitamin A deficiency, as inflammation causes RBP4 and TTR

to be temporarily suppressed. RBP4, the main transport protein for vitamin A in the blood is typically a sensitive marker to changes in nutritional and physiologic condition due to its short half-life (0.5 to 3 days) and regulated synthesis (Goodman, 1984; Robbins, 2002).

Conversely, results obtained in children with Type 1 diabetes have shown only a reduction in serum concentrations of retinol, but not RBP4 in comparison to healthy controls (Baena et al., 2002; Hozumi et al., 1998). One such case-control study in children completed in 2002 reported normal RBP concentrations in the presence of insulin with lower retinol levels (Martinoli et al., 1993). Consistent with reports in adults, the children in the study group showed signs of insufficiency (<0.20 mg/ml) or deficiency (<0.10 mg/ml) of vitamin A, with low-normal values ranging from 0.61 – 1.67 μg/ml. The decrease in retinol found in Type 1 diabetic children has been suggested to be the result of increased use of vitamin A as an antioxidant to eliminate free radical stress; however this theory remains unproven. In Type 1 diabetic children, research groups have found an elevation in CRP, paired with a decrease in available retinol, without a decrease in RBP4 and TTR (Espe et al., 2007). These results further ascertain that vitamin A deficiency, which is characterized by decreased retinol and RBP4, and normal TTR is not a cause in children with Type 1 diabetes. Instead, these studies show that RBP4 and TTR levels remain consistent in the presence of increased CRP in diabetse, suggesting that some other mechanism in Type 1 diabetes rather than inflammation is responsible for decreased serum retinol. In other words, an unknown mechanism impairs holo-RBP4 causing sub-optimal serum vitamin A.

To complicate matters more, recent research into Type 2 diabetes, obesity, cardiovascular diseases, and inflammation demonstrates that participants present with elevated RBP4 levels (Yang et al., 2005; Yao-Borengasser et al., 2007). Cell culture work has also found that elevated RBP4 concentrations may induce apoptosis, which may relate

with why humans with renal dysfunction and even in diabetic patients with microalbuminuria have increased RBP4 levels (Raila et al., 2007). To fill the gap in knowledge about vitamin A status in Type 1 diabetes, studies following changes in retinol, RBP, TTR and CRP *in vivo* from onset to adulthood are needed. Even if circulating serum retinol concentrations fall in the low-normal range with no overt vitamin A deficiency signs or symptoms, impaired immunity, wound healing and increased risk of infection have been long reported as manifestations (International Diabetes Federation, 2010).

Serum retinol half-lives are long, from 56-243 days and have been shown to not be relatively stable in the absence of disease (Burri and Clifford, 2004; Lemke et al., 2003; Rock et al., 1992). In diabetes, streptozotocin-induced murine models supplemented with vitamin A as retinyl palmitate also did not improve low-normal serum levels of retinol, RBP4 or TTR that were found (Lu et al., 2000b). Animal studies suggest that hepatic RBP mRNA expression and hepatic retinyl ester hydrolase activity may be altered, decreasing the availability of hepatic reserves for mobilization of vitamin A into the blood (Chen et al., 2003; Tuitoek et al., 1996a; Tuitoek et al., 1996b). This suggests another barrier preventing ingested vitamin A from being utilized by extra-hepatic tissues, and another justification of why it is worthwhile to explore the systemic effects of carotenoid supplementation in Type 1 diabetes.

Overall these studies suggest that there is abnormal metabolism of vitamin A in Type 1 diabetes, with decreased availability to peripheral organs due to decreased hepatic mobilization of retinol, yet the mechanism remains to be fully understood. Normalization of holo-RBP4 plays a key role in nutritional intervention and normalization of vitamin A status. As retinol supplementation failed to normalize plasma levels of RBP, alternate therapeutic strategies, including the effects and mechanisms of pro-vitamin A carotenoid supplementation still remain to be determined.

2. Vitamin A supplementation

In Type 1 diabetes, few studies have examined the long-term relationship between liver and plasma levels of provitamin A carotenoids, vitamin A and its carrier proteins with or without supplementation. Biobreeding diabetic prone (BBdp) rats spontaneously developing Type 1 diabetes have been supplemented with retinyl palmitate (18.5 μg/g/day) as part of the diet either alone or in combination with zinc until diagnosis of Type 1 diabetes (Lu et al., 2000a). No effect was found on plasma retinol or RBP4 concentrations, which remained sub-optimal, however hepatic RBP4 mRNA was significantly elevated. These results contrast murine models genetically lacking RBP that display low plasma retinol and vitamin A deficiency, with impaired vision in the absence of continuous dietary vitamin A which is transported via chylomicrons. In other words, continuous vitamin A supplementation treatment corrects deficits in RBP genetically lacking models, but does not correct low RBP levels. Study is required to determine what effect diabetes may have, and how well vitamin A maybe be utilized if lower levels of its primary transporters RBP4 and TTR are the primary concern. To date, no trials have considered the effects of β-carotene supplementation on metabolism alone; Randomized clinical trials have only assessed combination therapies of antioxidants (Dene et al., 2005; Hammes et al., 1997; Kowluru et al., 2003; Kowluru et al., 2008a; Miranda et al., 2004; Rytter et al., 2010).

Recent human and animal study of vitamin A supplementation in human Type 1 diabetes has been considered from the vantage of cardiovascular disease complications (Nicolle et al., 2003; Shidfar et al., 2010). Forty-eight, 7-year-old to 20-year-old patients with at least 2 years of Type 1 diabetes history, without any metabolic condition or insulin treatment participated in a randomized double-blind clinical trial for 12 weeks. With division into zinc and high dose vitamin A supplementation (10 mg elemental zinc per day and one-half of a 25,000 IU vitamin A tablet every other day) and a placebo group (oil only), a significant increase in apoprotein A-I, decrease in apoprotein B and total ratio of

apoprotein A-I: B were noted at the end of the study. Although unrelated to diabetic retinopathy, this provides novel information on vitamin A tolerance over a short duration, and some indication of an association between improved cardiovascular risk factors with vitamin A supplementation in a short-term Type 1 diabetic situation. The animal supplementation trial utilizing carrot powder (15%, w/w diet) found cardiovascular benefits, by reducing cholesterol absorption and susceptibility to lipid peroxidation over longer duration (Nicolle et al., 2003). Whether these high doses of vitamin A or carrot carotenoids also benefit retina function in diabetes requires investigation.

In healthy animal models it is well-known that vitamin A supplementation correlates well with liver levels of vitamin A, however serum levels remain relatively stable except in extreme deficiency or toxicity making it a poor marker of total body vitamin A reserves (Duncan et al., 1993). Borderline low vitamin A status in Type 1 diabetes has no overt symptoms or signs. Many studies had no means of determining status/deficiency as criterion, particularly in light of the unreliability of serum levels which remain constant regardless of status. Consequently, where vitamin A has been used as part of an antioxidant combination, there is a need to examine its effects independently and consider baseline vitamin A status and variable metabolic demand, in healthy versus Type 1 diabetic individuals. Animal studies should take in to account the longer term status in a variety of tissue and organ systems. This is particularly important in light of recent studies of β-carotene supplementation which have found a pro-oxidant effect with high doses in environments of high oxidative stress, as seen in smokers and asbestos-exposed workers (Omenn et al., 1991).

Carotenoids in disease

β-carotene has long been considered a non-toxic alternative to vitamin A supplementation. Consequently, β-carotene supplementation achieves conversion to only

strict levels of vitamin A in health. Meanwhile excess β-carotene is converted to other metabolic carotenoid byproducts. As a result, the optimum dose of β-carotene to achieve sufficient vitamin A has yet to be established in disease conditions.

Epidemiological findings suggest an inverse correlation between incidences of cardiovascular disease and increasing levels fruit and vegetable consumption, however supplementation studies have not yielded a protective effect (Gillman et al., 1995; Knekt et al., 1994). Randomized clinical trials looking specifically at the role of β-carotene have had mixed results, either finding no difference in cardiovascular incidence (Hennekens et al., 1996), reduced death from myocardial infarction (MI,44%) and stroke, vascularization and cardiac death (49%) in men with angina, increased risk of mortality in heavy smokers (Greenberg and Sporn, 1996; Liu et al., 2009). Results of the literature vary due to supplemental dosage of β-carotene, short study duration in populations, and effects which may vary with health condition. A pro-oxidant mechanism, increasing risk of lung cancer has been confirmed in heavy smokers and chronic alcoholics, contraindicating both vitamin A and high dose β-carotene supplementation in these select populations (Greenberg and Sporn, 1996; Liu et al., 2009).

In diabetes, a disease state characterized by high levels of oxidative stress, little is known about the systemic effects of carotenoid supplementation. In the absence of supplementation, vitamin A insufficiency paired with elevated serum β-carotene levels have been observed in some human and animal research. Lowered hepatic, but not intestinal conversion rate of β-carotene to retinol at comparable dietary intakes has been identified, but extrahepatic concentrations have not been extensively explored (Krill et al., 1997; Tuitoek et al., 1996a).

Carotenoid supplementation research

Healthy animal models

Healthy animal supplementation trials form the basis of existing β-carotene and α-carotene literature. Similar to vitamin A, β -carotene baseline absorption and metabolism has been assessed in several healthy animal models including Mongolian gerbils (Escaron and Tanumihardjo, 2006), ferret and rats (Ribaya-Mercado et al., 1989) at a variety of supplementation levels. In one existing study of a healthy rat model, no β-carotene was found in liver or adipose tissue suggesting complete conversion to vitamin A at the point of absorption. However this is likely the result of former methodological limitations, as more recent work, including our own reported here, have found the presence of both carotenoids in liver tissue (Goswami et al., 2003).

β-carotene bioavailability and bioconversion to vitamin A with a single oral pharmacological dose in oil or aqueous dispersion (0.15 μmol) rendered very low vitamin A conversion at levels less than 5%. Absorption and conversion to vitamin A did not differ among rats of different vitamin A states, indicating that a single, very high dose is not an effective strategy for increasing vitamin A in healthy animals (Goswami et al., 2003). With consideration to moderate doses of β-carotene in promotion of vitamin A sufficiency, a positive linear relationship has been noted in murine models between the intestinal absorption and the dose administered, with no saturation level up to 1440 μg. Small amounts of newly absorbed β-carotene were found in the liver, but not as isotopically labeled retinyl esters, suggesting inefficient enzymatic conversion of β-carotene, in situations of vitamin A sufficiency (Chen et al., 1999). Similar results have been found in studies supplementing varying levels of retinyl palmitate in conjunction with β-carotene suggesting a hepatic regulating mechanism exists in healthy state (van Vliet et al., 1996).

Over the last 5 years, gut-specific transcription factor ISX has been identified as a direct, independent repressor of β-carotene and α-carotene conversion to retinol (Seino et al., 2008). When cellular retinoid levels are elevated, there is repression of SR-BI and

BCMO1. This feedback mechanism explains why hypervitaminosis A toxicity does not occur with upper limit carotenoid supplementation, even in diabetes, where there may be poor carotenoid conversion and vitamin A sufficiency (Seino et al., 2008). This may also in part, explain carotenodermia, a dermal accumulation of carotenoids causing a yellow to orange colored skin associated with elevated carotenoid consumption, which has been reported with relatively low health risk (Mathews-Roth, 1988).

Perhaps some of the most interesting research comes from the recent discovery that unlike previously believed, β-carotene dioxygenase 2 (BCDO2) which cleaves xanthophylls and carotenoids asymmetrically, actually plays a role in the mitrochodria to protect cells from carotenoid induced dysfunctions (Amengual et al., 2011). Both BCDO2 hetergozygous and homozygous deficient animals supplemented with carotenoids (0.5mg/g lutein or zeaxanthin) accumulated triacylglycerides and developed liver steatosis with large lipid droplets. This was coupled with mitochondrial dysfunction manifested as oxidative stress. Phenotype was not seen in BCDO2 (-/-) knockout animals raised on chow diet without carotenoid supplementation. Increased oxidative stress denoted by manganese superoxide dismutase (MnSOD) was 9-times greater in the liver (Amengual et al., 2011). Previous clinical trials, including carotenoid supplementation in smokers and asbestos-exposed workers further suggest there may be a role whereby in certain conditions bypass the protective or non-toxic effects of carotenoids, triggering damage (Albanes et al., 1996; Omenn et al., 1994). Knockout of the carotenoid cleavage genes, heavy smoking, and pharmacological doses may be a few of these situations, however this remains to be proven.

In recent studies of healthy humans and monkeys, carotenoid supplementation has been shown to significantly increase levels of their metabolites in serum and ocular tissues (Rosales et al., 1996). Various carotenoid metabolites such as 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 have been identified in human eye tissues (Bernstein et al., 2001; Khachik et al., 1997). Additionally, many other cleavage products are believed to exist, but have not yet been identified due to lack of existing methods. In vitro, other researchers have reported that the oxidation of β-carotene also yields a series of products that result from the oxidative cleavage of the acyclic side chain, leading to the formation of a series of aldehydes which are highly reactive and capable of triggering cellular damage (Dillon et al., 2004). Authors speculate that possible antioxidant effects of carotenoids may be overwhelmed by oxidative stress in the retina of the AMD patients and therefore, a preventive role of carotenoids cannot be established by a number of epidemiological studies. Very little is known about modified products, including hydroxylated carotenoids, epoxides and rearrangement products and whether these products have biological activity. After lengthy exposure to oxidative stress, these alternative pathways may become important and result in an increase in biologically active derivatives. How carotenoid metabolites in ocular tissue impact diabetic retinopathy is unknown in vivo. Consequently, the understanding the role of these diverse carotenoid-derived products in the retina is necessary to determine the therapeutic value of carrot powder in retinopathy.

Type 2 diabetes & other disease states

In observational Type 2 diabetes research, Akbaraly et al. (2008) investigated the association between total serum carotenoid levels and 9-year onset of disturbances of glucose metabolism in a previously healthy elderly population (impaired fasting glucose or Type 2 diabetes). Risk of dysglycemia was significantly lower in participants with total plasma carotenoid levels in the highest quartile compared with participants in the lowest

quartile when controlled for extraneous variables. Intuitively, this suggests, that as there is already reduced conversion of carotenoids in diabetes, low dietary intakes of carotenoids could further exacerbate the problem from both a visual cycle metabolite and antioxidant perspective.

Similarly a 7.5-year randomized double-blind placebo-controlled fasting serum glucose study with combination antioxidant supplementation of 6 mg β-carotene in conjunction with 120 mg vitamin C, 30 mg vitamin E, 100 µg Se, and 20 mg Zn or a placebo, showed no significant difference after age-adjustment following intervention (Czernichow et al., 2006). At baseline, an inverse association between high β-carotene dietary intake, high β-carotene plasma concentrations and low fasting plasma blood glucose was noted, suggesting that β-carotene may have acted as an indirect marker of dietary fruit and vegetable intake by participants prior to study onset.

Conversely, the α-tocopherol, β-carotene Cancer Prevention Trial (ABCT), a double-blinded, randomized trial of 29,133 middle-aged male smokers showed no benefit towards cancer prevention, with a pro-oxidant effect in heavy smokers (Kataja-Tuomola et al., 2010). Either vitamin E 50 mg/day, β-carotene 20 mg/day, or both, or placebo was provided as supplements for a median of 6.1 years. At base-line, 1700 men had Type 2 diabetes mellitus. Of these men, 662 were diagnosed with their first-ever macro/microvascular complication, and 1142 died during the 19-year follow-up. Researchers concluded that neither supplementation affected the risk of macro/microvascular complications or total mortality.

Limitations of both studies are their lack of control over dietary intakes and use of infrequent food recalls, which even when administered, regularly lack accuracy. The methods employed were unable to account for changes in dietary consumption and seasonal variation over long duration. Considering the nutrient complexities of different

consumed food sources, and overall variability in dietary patterns between individuals, further in-depth study is warranted. Studies which control intakes would provide valuable information on the relationship between diabetes and vitamin A and carotenoid status. Compliance is another key concern in human studies; as demonstrated by Czernichow and colleagues (2006), one benchmark for compliancy and inclusion in their study was consumption 2/3 of capsules after 7.5 years, which in itself provides quite a large range of supplementation. Authors attributed discrepancies between their findings and those of other studies to their administration of lower doses of antioxidants, bias due to a better dietary pattern of enrolled volunteers and/or causal interference from post hoc analyses of the randomized trial which was not designed to test the role of antioxidants on glucose metabolism. In addition, as the study was limited to blood samples, plasma retinol was used even though it is thought to be a poor marker of body vitamin A reserves, and β-carotene plasma levels which were tracked are known to be affected by the disease. Furthermore, supplemental vitamin combinations that have included β-carotene are not translatable to actual food sources.

Diabetic retinopathy

Diabetic retinopathy, the leading cause of adult blindness, is perhaps the most debilitating of the microvascular complications (Jawa et al., 2004). Most individuals with Type 1 diabetes will exhibit some degree of retinopathy over the course of their lives. Duration of disease is a key factor in predicting rate of retinopathy, but is not the sole determinant of progression. Sixty % will develop proliferative retinopathy (Jawa et al., 2004), with progression to blindness in approximately 23% of all Type 1 individuals (Congdon et al., 2003). This remains a major challenge and suggests additional therapeutic management strategies are needed.

Neuroretinal dysfunction in diabetes has been widely studied in humans and animals. The most common sight-threatening lesion in Type 1 diabetes is the result of proliferative diabetic retinopathy (PDR), which is characterized by neovascularization (Joussen et al., 2007; Tong and Vernon, 2000). In spite of observational associations, and basic science research investigating diabetic retinopathy under hyperglycemic conditions, the pathogenesis is still not completely understood, making it challenging to determine an appropriate treatment strategy.

Major changes in diabetic retinopathy

Biochemical changes

Although there are fundamental gaps in knowledge existing about what mechanisms trigger damage to the retina in diabetes, investigations into the pathophysiology of Type 1 have identified irregularities in nutrient transportation, oxidative protection and visual cycle substrates in the retina. Consistent with altered vitamin A serum transport, vitamin A metabolism may be deregulated in the retina. Inter-photoreceptor retinoid binding protein (IRBP), the key carrier of fatty acids and retinal to and from retinal pigment epithelium and the rod outer segment for the visual cycle has been shown to be suppressed and associated with neurodegeneration (Simo et al., 2010). Provided the literature, it is conceivable that retinopathy precipitated by diabetes could be intensified by sub-optimal vitamin A via at least two mechanisms: a reduced access to distribute hepatic reserves of vitamin A to retina and a reduced vitamin A cycling within retina. In such a case, it is unknown if retinol converted from β-carotene and α-carotene supplementation, or their metabolites could play a role in ensuring sufficient, appropriate substrate in the pathway of rhodopsin generation and maintenance of visual health.

Electrophysiological changes

Only a handful of studies have measured the electrophysiology of the retina in human diabetes in order to determine its functionality. The electroretinogram (ERG) is a useful tool to measure the electrical responses generated by eyes, as the flash stimulus hyperpolarizes photoreceptors and other cell types. ERG recordings can be used to detect an inner retina dysfunction as reflected by abnormal OPs, before the presentation of clinical signs. The ERG finds inner retinal dysfunction proceeds before the clinical signs and symptoms of diabetic retinopathy, often being predictive in the development of vascular complications (Han et al., 2004; Aylward and Billson, 1989; Bui et al., 2003; Phipps et al., 2006). Following dark adaptation, the electroretinogram measures a-wave, primarily reflecting light-induced hyperpolarization of rod and cone (at light levels exceeding their threshold, which is higher than for rods) photoreceptor cells, and transducin cascade responses, and b-wave, bipolar cell membrane depolarization. Cone photoreceptor cell and the transduction cascade response are then tested in the presence of a light adapting background, whereby cone-driven b-wave and OPs also reflect dysfunction.

Experimental animals have more recently been employed to mimic functional and morphological retinal changes observed in human patients with diabetic retinopathy. The most commonly used rodent model of retinopathy is the streptozotocin (STZ)-induced diabetic rat, a model of Type 1 diabetes. STZ-induced rat models have found photoreceptor function to be altered at approximately 12 weeks of diabetes with low insulin administration (2 IU as needed) likely reflecting a decrease in the amount of rhodopsin present in the rod outer segments (Phipps et al., 2004). Rod and cone abnormalities, which have been related to apoptosis and glial dysfunction, which present as ERG inner retinal dysfunction, have been well established in diabetic animal models

and humans as an early sign of retinal changes; however mechanisms are not completely understood.

Oxidative stress

Although limited information exists on the explicit cause of diabetic retinopathy, the excessive formation of reactive oxygen species (ROS) in diabetes are also considered to be directly related with the pathophysiology of diabetic retinopathy. Light striking the retina is necessary to initiate the cascade of chemical and electrical events that ultimately trigger optic nerve impulses; however this can also be particularly problematic in the presence of highly enriched polyunsaturated lipids and cholesterol esters available to produce toxic oxidized products in the retina. Additionally, oxidative stress is tied to apoptosis in a variety of cell types, and is considered to be part of fundamental formation of diabetic retinopathy.

Healthy retinal tissue is capable of deactivating oxidators through a series of pigments (melanin, lipofuscin, glutathione), enzymatic (superoxide dismutase) and non-enzymatic antioxidants (lutein, zeaxanthin, ascorbate). Diabetic retinopathy in the presence of hyperglycemia has been observed to reduce the levels glutathione, superoxide dismutase, and ascorbic acid, creating an environment favoring oxidation and oxidation end products, and subsequently compromising retinal tissue and vision function (Simo et al., 2010). Streptozotocin-induced animals have also revealed elevated levels of glial fibrillary acidic protein (GFAP), a marker of inflammation reactivity in retinal Müller cells during Type 1 diabetes progression (Yee et al., 2010).

Oxidatively modified DNA is a reliable indicator of oxidative damage. Increased levels of 8-Oxo-2'-deoxyguanosine (8-OHdG) in the retina are implicated in the development of early diabetic lesions in retinopathy (Alper et al., 2005). Peroxynitrite, a marker of oxidative stress in the retina, produced from reaction between nitric oxide and

superoxide anion can also be used to establish the efficacy of a treatment. In early-onset diabetic retinopathy, high levels are present. It is well established in supplemental trials that treatments which inhibit apoptotic enzymes also decreasing retinal nitrative stress, as indicated by peroxynitrite (Kanwar et al., 2007; Kowluru and Odenbach, 2004; Kowluru et al., 2008a).

One recent study has suggested that complex III, an enzyme responsible for release of superoxide at the mitochondrial membrane is downregulated in Type 1 diabetes, and is one mechanism for diabetes-induced increased retinal superoxide (Kanwar et al., 2007). In streptozotocin-induced murine models supplemented with antioxidants, upregulation of MnSOD helped defend against the increase in retinal oxidative stress caused by the superoxides (Kowluru et al., 2008b). This suggests a role that β-carotene may participate as an antioxidant and scavenger to counteract oxidative stress, further triggering vitamin A chromophore depletion in the visual cycle, but remaining to be tested. Therapeutic strategies incorporating antioxidant co-treatment in conjunction with β-carotene and vitamin A have been found to defend against ROS in the retina (Dene et al., 2005; Hammes et al., 1997; Kowluru et al., 2003; Kowluru et al., 2008a; Miranda et al., 2004; Rytter et al., 2010). Conversely, β-carotene which acts as scavenger, may be ineffective in an environment of oxidative stress (ie smoking studies, asbestos) as it has the potential to also be oxidized in the retina.

Concurrently, retinoids such as retinol, are believed to exert a protective effect against neurodegenerative and cardiovascular diseases by acting as an antioxidant and maintaining cellular redox homeostasis by deactivating ROS produced in metabolism (Gutteridge and Halliwell, 1982). However, findings are mixed, with a wave of clinical trials in the 1990s which found a pro-oxidant action of retinol and other retinoids in conditions with high oxidative stress, such as cancer and cardiovascular disease (Omenn et

al., 1994). As a result, additional clarification to the role of vitamin A, and specifically β-carotene as antioxidants are required to clarify their effects on redox homeostasis within the retina in diabetes.

Advanced glycation end products

In diabetic retinopathy advanced glycation end products (AGEs) and Amadori reaction adducts have been localized to retina neurons, glia and vascular cells (Gardiner et al., 2003; Stitt et al., 1997). For this reason, AGE adduct levels produced in the presence of carotenoid and vitamin A supplementation, compounds which are highly reactive with oxidants, may reflect changes to inner and outer retinal function.

Produced from compounds with reactive aldehyde groups such as Amadori adducts, AGEs undergo a series of glycation and oxidation reactions between reducing sugars and available free amino groups on proteins, lipids and DNA available in the environment (Baynes, 2001; Schmidt et al., 1995). AGEs are stable within physiological conditions, and depending on the initial components may cause fluorescence, pigmentation, damage by impairing receptors, cross-linking and altering enzymatic activity (Stitt, 2010). Recently, the injection of preformed AGEs bound to albumin triggered damage to the retina of healthy animals (Stitt et al., 2000). These animals were found to have breakdown of the blood-retinal barrier, with basement membrane thickening, lower levels of nitric oxide sythetase and vasoregulatory abnormalities, which all contribute to the pathophysiology of diabetic retinopathy. Moreover, AGE-R1 which degrades AGEs has further been found to have diminished expression in severe Type 1 diabetic complications, leading to elevated serum AGE levels (He et al., 2001).

Methylglyoxal-imidazolone and N-$^{\varepsilon}$(carboxymethyl) lysine (CML) are predominant AGE adducts that have been found in the retina and plasma of diabetic humans and murine models (Hammes et al., 1999). CML (a non-fluorescing AGE) and

other AGEs have been localized to retina vasculature in patients with type 2 diabetes with varying degrees of retinopathy (Murata et al., 1997). Methylglyoxal-derived hydroimidazolone has been shown to increase by 279% in 24-week diabetic rat retina and concentrated within Müller cells (Karachalias et al., 2003). Both AGEs are thought to be a consequence of deposition of circulating AGEs and local synthesis, however they have not been considered with carotenoid supplementation. It is suspected that hypoxia and excess lactic acid, paired with methylglyoxal concentrations found to be 5-6 times greater in Type 1 diabetes, make hydroimidazolone a major glycating agent in diabetic patients (Thornalley, 1998). Hyperglycemia induces overproduction of superoxides by the mitochondrial electron transport chain, which is suspected to trigger numerous AGE adducts exacerbating pathogenic response (Brownlee, 2005; Hammes, 2005). Other AGEs come from glycolytic metabolism, such as N-(carboxyethyl) lysine (CEL) and carboxymethyl-arginine (CMA), and are synonymous with serum high-glucose exposure, a parameter of diabetes, with many more that have not yet been characterized (Thornalley et al., 1999).

Dyslipidemia, which may also be present in the absence of insulin control in the Type 1 patient population is another root of adducts formed in the lipid rich retina environment, by the Maillard reaction. In fact, high accumulation of polyunsaturated lipids in transgenic *fat*-1 mice retina showed an increase of carboxyethylpyrrole, an adduct that forms from the oxidation of docosahexanoic acid (Suh et al., 2009). Such products are classified as advanced lipidoxidation endproducts (ALEs). ALE N-$^{\varepsilon}$(3-formyl-3,4 dehydropiperidino) lysine (FDP-lysine) has been associated with severity of retinopathy in both Type 1 and Type 2 diabetic individuals (Zhang et al., 2008). ALEs have also not yet been considered with carotenoid supplementation in spite of their close association with lipid transport and metabolism.

Lipofuscin, pigmented granules formed of toxic byproducts including oxidized protein and lipid residues, along with all-*trans*-retinal are found as deposits in the retinal pigment epithelium in diseases of progressive retinal degeneration, yet there are very few reports in diabetic retinopathy. During photobleaching of rhodopsin, photoproduct exists in millimolar concentrations within the cell. During this time, slow conversion or high concentrations of chromophore can allow aldehyde groups to form adducts with the primary amino groups that exist in many molecules including lipids, proteins and ribonucleotides (Stitt, 2010). Aberrant reactions of all-*trans*-retinal with membrane phosphatidylethanolamine, form bisretinoid N-retinyl-N-retinylidene ethanolamine (A2E). Coupled with impaired clearance, lipofuscin deposits containing A2E are implicated in the pathogenesis of age-related macular degeneration (von Lintig et al., 2010). Mechanisms by which photoproduct induces photoreceptor cell death remain controversial (Maeda et al., 2009). What impact high dose β-carotene supplementation will have on the availability of all-*trans*-retinal is unknown in diabetes. It is conceivable that in the presence of diabetic suppression of IRBP transporter, carrier of fatty acids and retinal, there may be an unexplored effect on retinal vitamin A metabolism and development of adducts.

Present challenges of AGE and ALE measurement are the lack of standardized methods and consensus on biological relevancy, making it difficult to compare results between studies, and between blood and tissue findings. Other AGE variations in the literature may be attributable to non-uniformity of assays, patient population variation, and presence of nephropathy causing poor clearance of AGEs from the blood stream. One recently published non-invasive method of measuring AGEs is by reading skin autofluorescence (AF) which is applicable to some, but not all AGEs. In a study of Type 1 patients, there was tissue accumulation of AGE which significantly trended with microvascular complications; however nothing is known about the impact of carotenoid supplementation (Araszkiewicz et al., 2011; Goh and Cooper, 2008).

Receptors of advanced glycation endproducts

Alternate measurement can be achieved with receptors of advanced glycation end products (RAGE), from the Ig receptor family. A variety of ligands, including AGEs are capable of binding to the RAGE cell surface receptor, inciting inflammation and progressive vascular injury through translocation of binding element, such as NF-κB, and the upregulation of RAGE itself (Li and Schmidt, 1997). In vitro, high concentrations of retinol have also been shown to induce the activation of redox-sensitive pathways which result in the up-regulation of RAGE in cultured Sertoli cells, a cell type with endrocrine function and physiologic target of vitamin A (Gelain et al., 2011). The same research group has also observed that retinol at high concentrations, is able to enhance reactive species production and induce extensive oxidative damage in cultured Sertoli cells (Dal-Pizzol et al., 2001; Pasquali et al., 2008). RAGE activation is known to participate in a wide range of pathological conditions, including diabetes, and other pro-inflammatory states where the gradual accumulation of AGE was observed to trigger signaling responses in the mitochondria of cells (Yan et al., 1997). Receptors are localized to glia and inner retina, particularly Müller cells. Recently abnormal expressions of sRAGE and esRAGE have been demonstrated in Type 1 and Type 2 diabetes and associated complications (Kalea et al., 2009). Consequently, this may suggest that RAGE induced by retinol could predispose cells to further damage and more RAGE ligands. The impact of β-carotene supplementation on retinol conversion in Type 1 diabetes, for these reasons requires investigation.

Anti-oxidant and known RAGE inhibitor co-treatments in the presence of retinol were observed to reverse the induction in RAGE and reactive oxygen species suggesting a predominant role for oxidative stress and protein kinase activation of p38 and Akt in the process (Gelain et al., 2011). A STZ-induced C57BL/6J mouse model recently found that

inhibition of RAGE by pharmacological agents blocked the development of important lesions of diabetic retinopathy, suggesting the importance of minimizing RAGE activation (Li et al., 2011). However, in vivo study and immunocytochemistry are still ultimately required to confirm the role of retinol concentration on RAGE activation. The effect of β-carotene in such an environment is unclear as it has not yet been tested. Subsequently, it is unknown whether β-carotene would readily undergo conversion to retinol, prevent oxidation as an anti-oxidant, or drive oxidation damage in diabetes activating RAGE.

Other parameters altered by Type 1 diabetes

Secretory factor levels found in the retina are also known to be affected in proliferative diabetic retinopathy (PDR). Among them, vascular endothelium growth factor (VEGF) which usually appears in low concentrations in normal state, is unregulated by hypoxia, as is erythropoietin, which may contribute to angiogenesis, and ApoA1, which has an unknown role. Meanwhile pigment-epithelium derived factor (PEDF), the protector from hypoxia induced stress is downregulated. Much has been hypothesized about these compounds, but their role in the progression of PDR has yet to be fully elucidated. More recently, Bruclacher and colleagues (2008) found that in contrast to previously held knowledge, diabetes-related endothelial injury of the retina could be a result of hyperglycemia-induced cytokine release by retinal cells, and not the direct effect of high glucose levels in the tissue. Altered signaling of protein kinase C (PKC) and nuclear factor kappa-B (NF-kB), MAP kinase phosphorylation have been seen to damage retinal endothelial cells, pericytes, neurons, glia and pigment epithelial cells and recruit inflammatory cells to produce vasoactive compounds, growth factors, coagulants leading to angiogenesis and tissue remodeling in diabetic retinopathy (Pelikanova, 2007). Future studies should consider these affected parameters. Studies continue to show that people living with Type 2 diabetes mellitus related eye complications, including cataracts, tend to

have borderline sufficient levels of serum β-carotene (Rytter et al., 2010; Brazionis et al., 2009). However, little is known of the relationship between carotenoids and diabetic complications. The relationship between diabetic eye conditions and sub-optimal vitamin A and provitamin A carotenoid levels requires investigation, as little research concerning β-carotene and overall carotenoid status of the retina in Type 1 diabetes exists.

Morphological changes

Histological analysis from a STZ-induced murine model investigation found no significant loss of photoreceptors during Type 1 diabetes or experimental polyunsaturated fatty acid diet treatments (Yee et al., 2010). It should be noted, that no quantitative analysis of rhodopsin or cone opsin levels was completed in this study, and consequently there is the possibility that the level of opsin expression may have been altered, but not accounted for.

In a 2010 comparative study using dual wavelength auto-fluorescence imaging, Type 2 diabetic patients with or without clinical diagnosed retinopathy had reduced macular pigment optical density (MPOD) compared to healthy controls. An inverse correlation between MPOD and HbA1C levels was also observed. In other words, macular pigment consisting of carotenoids lutein and zeaxanthin deteriorates with poor glycemic control and oxidative stress. In observational studies, Type 2 diabetic patients with a higher lutein and zeaxanthin to provitamin A carotenoid ratio predicted lower risk of developing diabetic retinopathy after adjustment for confounders (Brazionis et al., 2009). In essence, higher concentrations of β-carotene and α-carotene to lutein and zeaxanthin were associated with greater risk of developing diabetic retinopathy. It highlights the importance of complex carotenoid supplementation, rather than individual supplementation. Unfortunately, neither study has been reproduced for Type 1 diabetes. While diabetic macular edema is the primary cause of lesions in Type 2 diabetes, Type 1

diabetes remains similar in its pathologies including impaired oxygen transport with increased affinity of oxygen for glycosylated hemoglobin (Ditzel, 1976), abnormal vasculature of the retinal capillaries and a oxidative shift in glycolysis (Hilmantel et al., 1999) which are all potential contributors to impaired macular pigment density (Joussen et al., 2007).

Steptozotocin-induced rat models, have in some studies demonstrated reduced total thickness of retinas in Type 1 diabetes (Arnal et al., 2009). Specifically, the thickness of the outer and inner nuclear layers was reduced significantly in diabetic rats and demonstrated cell death in the ganglion cell layer which is comprised of ganglion cells and displaced amacrine cells, which may be involved in the generation of OPs. Interestingly, retinal changes were avoided with administration DHA and lutein alone in both hyperglycemic and normoglycemic (insulin-administered) conditions. Considered collectively, these observations suggest potential for dietary modulation of diabetic retinopathy risk through carotenoid supplementation.

Existing literature reveals at least two mechanisms of vitamin A and carotenoid metabolic impairment which are specific to vision. First, less vitamin A is accessible for visual cycle substrate formation. Second, loss of vitamin A and carotenoids as antioxidants in the eye reduce their ability to counteract oxidative stress. Two questions arise from these studies: will provitamin A carotenoid supplementation improve vitamin A availability?; will complex carotenoid supplementation confer any additional protective functions? Ultimate confirmation of the therapeutic effects of vitamin and provitamin A supplementation in the Type 1 diabetic state will garner knowledge of their synergistic effects.

Carotenoid supplementation in diabetic retinopathy

Antioxidant combinations routinely utilized in a clinical setting for age-related macular degeneration (AMD) may also play a protective role in diabetic retinopathy onset and progression, as well as with other disease associated abnormalities if in fact oxidative stress contributes to progression (Barker, 2010). As reviewed by Carpentier and colleagues (2009), potential benefits from supplementation in AMD and cataracts were shown with intermediate levels of lutein (10–15 mg/d) as well as high levels (>30 mg/d). Plasma concentrations increased with supplementation of at least 5 mg lutein and 0.3 mg zeaxanthin at a proportional level.

Previous observational data on the relationship between serum and plasma carotenoids and Type 1 and Type 2 diabetes has been acquired from community based, cross-sectional studies. Studies have found associations between diabetic retinopathy and serum and plasma carotenoid levels (Ford et al., 1999; Mares et al., 2006). Higher serum and plasma lutein and zeaxanthin to provitamin A carotenoids ratio predicted lower diabetic retinopathy risk (Brazionis et al., 2009; Dene et al., 2005). Meanwhile a 2001-2004 observational study of a cohort of 1698 women (53-86 years of age), examining macular pigment optical density (MPOD) by heterochromatic flicker photometry and dietary intake, found that MPOD was directly related to dietary intake of lutein and zeaxanthin but even more strongly to serum concentrations, which could reflect immeasurable factors influencing their uptake and utilization. Lower MPOD also corresponded to higher incidence of developing diabetes. These studies suggest that there is in fact some level of utilization linked with diabetes pathogenesis without negative consequences from dietary consumption.

Although a clear connection between the beneficial effects of carotenoids in diabetes has not been made, studies have demonstrated that β-carotene alone or combined

with other micronutrients prevented a diabetes-induced increase in the number of retinal acellular capillaries, increased levels of retinal glutathione peroxidase, which is downregulated in diabetes, and inhibited oxidatively modified DNA (Kowluru et al., 2008a). Nitrotyrosine and MnSOD were also downregulated in Type 1 diabetic murine models (Dene et al., 2005; Kowluru et al., 2008a).

Dene et al. supplemented STZ-induced Type 1 diabetic rats with β-carotene (10mg/kg/day), and were able to increase levels of retinal glutathione peroxidase, which is decreased in diabetes, promoting conversion of the antioxidant glutathione (Dene et al., 2005). Streptozotocin-induced diabetic rats received a powdered diet with or without supplemental micronutrients (ascorbic acid, vitamin E, β-carotene, zinc, and copper). 1.5 mg/kg/day of β-carotene in combination with the other micronutrients prevented a diabetes-induced increase in the number of retinal acellular capillaries. In the same rats, there was also inhibition of oxidatively modified DNA and nitrotyrosine, downregulation of MnSOD, downregulation of diabetes induced alterations in coenzyme Q cytochrome-c reductase and nitric oxide synthase (Kowluru et al., 2008a). These studies indicate that β-carotene may have potential to protect against retina pathology in diabetes. Three questions that remain to be answered from these studies are: will β-carotene supplementation from carrots reduce markers of oxidation in the retina when supplemented alone?; will β-carotene improve vitamin A availability for the visual cycle in the retina when supplemented alone?; are provitamin A carotenoids specifically involved in rod- and cone-function?

Table 1-1. Studies of diabetes associated with carotenoids and carotenoid supplementation

Classification	Subjects (# of subjects)	Results	References
Type 2 diabetes & impaired glucose	Human observational (n=655)	α and β-carotene, lycopene, lutein/zeaxanthin inversely associated with fasting serum insulin	Ford et al. (1999)
Type 2 diabetes	Human cross-sectional (n=111)	Lutein and zeaxanthin significantly lower than α and β-carotene in diabetic retinopathy	Brazionis et al. (2009)
Type 1 diabetes	STZ-induced rats β-carotene (10mg/kg body wt) administered alone or with pharmacological agents	β-carotene alone increased glutathione reductase. In combination, it increased superoxide dismutase in the retina	Dene et al. (2005)
Type 1 diabetes	STZ-induced rats β-carotene (1.5mg/kg body wt) in combination with ascorbic acid (50mg/kg), vitamin E (0.5mg/kg), zinc (8mg/kg) and copper (0.2mg/kg)	Combination therapy prevented acellular capillaries in the retina. Decreased oxidatively modified DNA, nitrotyrosine and MnSOD (inhibiting diabetic retinopathy by inhibiting oxidative stress)	Kowluru et al. (2008)
Type 1 diabetes	STZ-induced rats lutein (0.02% wt/wt), zeaxanthin (0.01% wt/wt)	Zeaxanthin inhibited retinal oxidative damage. Reduced VEGF and adhesion molecules associated with diabetic retinopathy	Kowluru et al. (2008)
Type 1 diabetes	STZ-induced mice 11-*cis*-retinal (25 mg/kg bolus)	Corrected outer retinal uptake, but not to control levels	Berkowitz et al. (2012)
Type 1 diabetes	STZ-induced rats lutein (0.5mg/kg) & DHA (13.3mg/kg)	Reduced oxidative stress in diabetic encephalopathy	Arnal et al. (2010)
Type 1 diabetes	STZ-induced mice lutein 0.1% (w/w)	Prevented reactive oxygen species generation in the retina and minimized visual impairment (as per ERG)	Sasaki et al. (2010)

Recently, supplementation of 11-*cis*-retinal (single dose 25mg/kg) significantly corrected outer retinal uptake, in diabetic mice, although not to control levels (Berkowitz et al., 2012). From this study it was not clear how retinal stress in diabetes is linked with rhodopsin regeneration, however it was suggested that supplementation may restore some level of manganese uptake required to open channels in the presence of sufficient ATP. No

studies have yet considered how pro-vitamin A may affect outer retinal uptake and ultimately vision.

Lutein and zeaxanthin are relatively new compounds of interest with regard to diabetic retinopathy and other vision disease supplementation studies. They are currently being examined in clinical settings for AMD and cataracts (Carpentier et al., 2009), and may also play a protective role in diabetic retinopathy onset and progression. Existing studies have supplemented these non-provitamin carotenoids both alone and in combination with antioxidant vitamins (Carpentier et al., 2009). They have not been examined in conjunction with α and β-carotene in supplemental form, nor from a food source.

Murine Type 1 diabetic models supplemented with lutein, zeaxanthin, or in combination with DHA have been shown to minimize ROS generation in the retina and to suspend visual impairment associated by diabetes when measured against indicators of oxidative stress (Arnal et al., 2010; Sasaki et al., 2010). It would have been of interest if they had assessed rod and cone function to show whether there were any functional improvement of retina.

Lutein (0.5mg/kg/day) administered orally for 12 weeks in STZ-induced diabetic rats was shown to restore diabetes-induced retinal alterations of glutathione concentrations and glutathione peroxidase activity to healthy levels. Restoration had a cascading effect which restored nutrient metabolism, gene expression of apoptosis, cytokine production, and VEGF to a level more consistent with a pre-diabetic state (Arnal et al., 2009). In another study, a diet supplemented with 0.02% or 0.1% (w/w diet) zeaxanthin soon after induction of diabetes prevented diabetes-induced increase in retinal damage, by increasing VEGF and intercellular adhesion molecule ICAM-1 (Kowluru et al., 2008). Zeaxanthin (0.02% and 0.1%) was found to have similar effects on diabetes-induced retinal

abnormalities, without improving hyperglycemia. Unlike lutein in the Arnal and colleagues study (2009), zeaxanthin administration failed to decrease in retinal glutathione concentration levels, suggesting that non-provitamin A carotenoids lutein and zeaxanthin have different beneficial effects on the retina in a Type 1 diabetic environment.

In C57BL/6 mice with streptozotocin-induced diabetes a lutein-supplemented diet 0.1% (w/w) with mouse chow prevented ROS generation in the retina and signal-regulated kinase activation (Sasaki et al., 2010).

Bioavailability of supplementation

Factors influencing the bioavailability and bioconversion are an equally important consideration when supplementing the diet with nutrient combinations. As most studies have focused on administering only one or two synthetic carotenoid compounds at a time, little is known about the synergistic or inhibitory roles carotenoid mixtures have on absorption, metabolism or action on markers of disease in animal or human systems. Studies on simultaneous ingestion of carotenoids in healthy models have had mixed results about the inhibition/promotion of absorption of non-provitamin carotenoids lutein and zeaxanthin in the presence of β-carotene. One study found lutein and canthaxanthin absorption to be inhibited in the presence of β-carotene (Maiani et al., 2009) . Conversely, another research group showed no interaction towards absorption by β-carotene, β-cryptoxanthin, α-carotene, lutein and zeaxanthin (Hoppe et al., 2003). More recently, a study of carotenoid absorption in Leghorn chicks found high dose of a single carotenoid alters absorption of other carotenoids (Wang et al., 2010). Capacity of the retina to accumulate lutein and zeaxanthin was diminished when β-carotene intake was high. Impact of food product, such as carrot rich in β-carotene and α-carotene on lutein and zeaxanthin remains to be determined.

In healthy human populations, there has been no major observed difference in bioavailability of β-carotene, α-carotene and lutein or isomerization during absorption and intestinal conversion (Cardinault et al., 2003). However as previously detailed, serum and plasma levels of retinol and other precursors are less reliable indicators of status than tissue levels which are unobtainable in human study. Evidently, more research is required to fully understand the true dynamic of carotenoid combination therapy.

A novel opportunity for further research would be to determine an effective provitamin A and non-provitamin carotenoid dose (in whole food or supplemental form) that delays the progression of diabetic retinopathy. As these compounds are found in nature in foods that are also high in pro-vitamin A carotenoids, research assessing the synergy of carotenoids in diabetic retinopathy prevention is needed. A determination of what levels of specific carotenoids might be suitable reduce the risk and progression of diabetic retinopathy is needed. In the present situation, the impact of provitamin and non-provitamin A carotenoids on diabetic retinopathy obtained from naturally enriched whole food requires investigation.

Carrot as a source of vitamin A and carotenoids

The carrot has been called a health food for the eyes for decades. Yet, no interventional studies have examined its effects in retinopathy of any kind. It is the leading dietary vegetable around the world providing approximately 14% of total vitamin A consumption and one of the richest sources of carotenoids, especially β-carotene, and α-carotene, with lesser amounts of lutein and zeaxanthin (Desobry et al., 1998). In addition, carrots contain fibre (2.4g/100g of raw carrot), which has been long found improve cholesterol, balance intestinal pH and slow the intestinal absorption and release of glucose, a key strategy in diabetic control.

In 1990, a study of 19 carrot cultivars observed levels of β-carotene between 1200 to 2300 mg per 100 g of raw carrot, which is considered to satisfy the human daily vitamin A requirement through bioconversion (Heinonen, 1990). According to Desobry et al. (1998) carrot carotenoids can be concentrated through reduction in water content and maintained for dietary supplementation when preserved by freeze-drying, and encapsulation (Desobry et al., 1998). Table 1-2 depicts the Health Canada nutrient file information for 100g of raw carrot (Health Canada, 2009b). Alpha-carotene may vary from 15 to 40% of total carotenoids, β-carotene from 44 to 79% (Desobry et al., 1998).

Table 1-2: Nutrient composition of raw carrot (100g)

Nutrient name	**Per 100 g**
Protein (g)	0.93
Total Fat (g)	0.24
Carbohydrate (g)	9.58
Energy (kcal)	41
Fibre, 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-cryptozanthin (µg)	0

Information adapted from Health Canada: Canadian Nutrient File (Health Canada, 2009b).

Numerous studies have used purified synthetic vitamin α-carotene, β-carotene, lutein, zeaxanthin alone and for supplementation; however, the therapeutic value of a whole food containing several carotenoids may carry greater rewards that remain to be tested. Interestingly, the influence of carrots on the eye in health has also never been

directly tested. It remains to be resolved if carotenoids found in carrot powder will affect vitamin A availability in the retina by altering vitamin A mobilization, and if their antioxidant characteristics will benefit in rod and cone function and vision in health and diabetes.

Chapter 2. RESEARCH PLAN

Rationale:

Diabetes mellitus, a metabolic disorder characterized by the presence of hyperglycemia due to defective insulin secretion, has been declared a major public health issue. Among the complications of the disease, retinopathy can have a significant impact on the mobility and life quality of individuals living with diabetes. As the leading cause of blindness in adults, 23% of all Type 1 and 14% of Type 2 diabetic individuals will become completely blind over their lifetime (Chaturvedi et al., 2001; Congdon et al., 2003). Duration of disease is a significant factor in predicting rate of retinopathy, but does not solely dictate rate of progression. Tight glycemic and blood pressure control, treatment of hyperlipidemia, and smoking cessation have had a demonstrated reduction in risk of developing microvascular complications; however retinopathy still develops in 12% of intensively treated diabetic patients (Chaturvedi et al., 2001). This remains a major challenge and suggests additional therapeutic management strategies are needed.

Vitamin A plays a vital role in visual function as a chromophore of photoreceptors in the retina for photoisomerization. To sustain visual cycle, continuous supply of this nutrient is essential. While vitamin A deficiency leading to night blindness is widely documented, limited information is available about its role in diabetes. Studies have shown abnormal metabolism of vitamin A in Type 1 diabetes, with decreased availability in the retina due to downregulated inter-photoreceptor retinoid binding protein and decreased hepatic mobilization of retinol and hepatic retinol binding protein (RBP4)

(Basu et al., 1989; Lu et al., 2000a; Tuitoek et al., 1996c; Simo et al., 2010). These studies indicate that low vitamin A status may worsen the visual cycle in diabetic retinopathy, but cannot necessarily be replenished with vitamin A supplementation alone.

Carotenoids are also necessary in vision as provitamin A and for ocular structure. Studies also show that people with diabetes having eye problems tend to have high-normal serum β-carotene, a precursor of vitamin A, and low retinal lutein and zeaxanthin, which are known as visual pigments (Brazionis et al., 2009; Kowluru et al., 2008). A clear connection between the beneficial effects of carotenoids in diabetes has not yet been established. Yet, studies have demonstrated that β-carotene alone or combined with other micronutrients (ascorbic acid, vitamin E, β-carotene, zinc, and copper) prevented a diabetes-induced increase in the number of retinal acellular capillaries, increased levels of retinal glutathione peroxidase, which is downregulated in diabetes, and inhibited oxidatively modified DNA (Kanwar et al., 2007; Kowluru and Odenbach, 2004; Kowluru et al., 2008a). However, there is no evidence that these positive changes are indeed linked with improvement of retinal function. Additionally, there has been no information about the use of carotenoids to overcome the decreased vitamin A levels found in diabetic metabolism. This study will investigate the involvement of carrot (a whole food) as a preventive or ameliorating therapy for the diabetic retina, as the disease remains neither preventable, nor curable.

Hypothesis: The working hypothesis of this research is that consumption of carrot powder will improve retinal function in Type 1 diabetes mellitus by increasing carotenoids and vitamin A bioavailability. More specifically,

Whole carrot powder will:

1. Improve retinal function in healthy and diabetic animals

2. Increase the availability of tissue carotenoids and retinoids

Objectives: The objective of this investigation is to determine whether a carrot product, specifically carrot powder, consumed in combination with food reduces the development of diabetic retinopathy.

This study will test the hypothesis with four primary objectives:

1. To determine if carrot powder improves the metabolic parameters of Type 1 diabetes
2. To examine if carrot powder influences the retina in health and the diabetes
3. To examine if carrot powder influences the bioavailability of carotenoids and retinoids in serum, liver and retina in health and Type 1 diabetes
4. To measure the effect of carrot powder on levels of retinol binding proteins in the liver and serum

Chapter 3. EXPERIMENTAL DESIGN AND METHODS

Experimental Design:

1. Diets & animals

All study protocols were approved by the University of Manitoba Office of Research Ethics & Compliance and Animal Care Committee. Three week old weanling male Wistar rats (Strain Code: 003) were purchased from Charles River Laboratories (St. Constant, PQ) and housed in pairs in flat-bottomed polycarbonate cages in a temperature and humidity controlled room with a 12-hour light dark cycle. After four day acclimatization, animals (n=30) were assigned to one of two diets; carrot enriched 15g/100g diet (Nicolle et al., 2003) or carrot free semi-synthetic control diet commonly used in nutrition studies and fed *ad libitum* (Table 3-1). Carrot powder for the diet was provided by the Food Product Development Centre (Portage la Prairie, MB, Canada). The nutrient contents of the powder were measured by SGS Canada Inc. (Vancouver, BC, Canada) and contained protein (8.2%), carbohydrate (74.9%), fat (1.8%), moisture (8.4%) and ash (6.6%). Mineral and fibre content were found to be approximately 3.3g/100g and 30g/100g respectively. These values were within a similar range to exisiting data (Health Canada, 2009b; Nicolle et al., 2003). Carotenoid content (β-carotene, α-carotene, lutein, and zeaxanthin) was quantified from the study carrot powder by reverse-phase ultra performance liquid chromatography (UPLC) with photodiode array (PDA) detection (Acquity, Waters Corp., Milford, USA) prior to study start in order to determine the quantity of carotenoids being supplemented in the diet (Table 3-1). The control diet was modified based on the carrot powder nutrient content and provided as approximately 35% of calories as fat, 42-44% carbohydrate and 20% protein, reflecting the current macronutrient distributions recommendations for by the Canadian Diabetes Association (2008). With this approach, diet induced changes in metabolism and function were

considered to be based on differences in micronutrients such as carotenoids, (β-carotene, α-carotene, lutein, 61.9 mg, 43.1 mg and 0.62 mg per kg diet after accounting for recovery). All animals received fresh feed three times per week to minimize ingredient decomposition and nutrient loss. The stability of carrot powder carotenoids in the food were also measured by leaving an extra cup beside the animal cage which was collected and measured.

Table 3-1: Composition of experimental diets (g/kg)

	Control Diet	Carrot Diet
Casein	218.00	216.00
Corn Starch	423.00	403.50
Sucrose	37.50	-
Glucose	15.00	-
Fructose	15.00	-
Non-Nutritive Cellulose	50.00	0.00
Vitamin Mix AIN-93VX	10.00	9.00
Mineral Mix AIN-93M	50.00	45.00
Choline Chloride	2.75	2.75
Inositol*	6.25	6.25
L-Methionine	2.50	2.50
Canola Oil**	170.00	170.00
Carrot Powder***	-	150.00

Diet ingredients were purchased from Dyets Inc. (Bethlehem, PA) except canola oil and Inositol
* Inositol (Bio-Serv, Frenchtown, NJ)
** Capri Canola Oil (Bunge Ltd, MB, Canada) containing dimethylpolysiloxane, as an anti-foaming agent.
*** Contains approx. 10.5g protein, 18g starch, 37.5g sucrose, 15g glucose, 15g fructose, 0.05g vitamins, 4.5g minerals, 47.5g fiber

After three weeks of feeding, animals in each diet group were fasted overnight before tail vein injection of either a vehicle solution (non-diabetic) or streptozotocin (Tocris Biosciences, Ellisville, Missouri, USA) at 65mg/kg body weight (0.2M acetate buffer, pH 4.5) I.V. to induce type 1 diabetes (D, DC n=8). In order to provide additional supportive hydration for recovery, a 2ml saline flush and 5ml intradermal injection of saline were also administered to each animal. The final experimental groups were as follows: i) Healthy

without carrot enrichment (N, n=7); ii) Healthy with carrot enrichment (NC, n=7); iii) Diabetic without carrot enrichment (D, n=8); iv) Diabetic with carrot enrichment (DC, n=8). Free access to water and diet were also provided following injection. After three days, animals with fasted blood glucose of 12mmol/L or greater were considered diabetic. In the case of animals who were not diabetic, a second injection of streptozotocin was provided as follows: 50mg/kg wt for blood glucose readings in the range of 5-7mmol/L, and 25mg/kg wt, for animals with values of 8-11mmol/L. Animals continued on their designated diets for a further 9 weeks to ensure development of diabetic retinopathy as indicated in the literature (Phipps et al., 2006). Dietary intake, body weight, fed blood glucose were monitored weekly. Insulin was not administered to diabetic animals in order to maintain elevated blood glucose during the study period.

2. Metabolic cages

On week eleven of the experimental period, seven or eight animals from each treatment were housed individually in metabolic cages for a three-day period beginning at 8:30 a.m. Twenty-four hour feed intake, water consumption, and feces weight were recorded, meanwhile urinary volume and collection occurred with storage at -80C until analysis.

3. Tissue collection

At the end of the 12 weeks, full field electroretinogram (ERG) was employed to measure retinal function, followed by intraperitoneal injection of pentobarbital (120mg/kg body wt) and cervical dislocation. Blood was collected by cardiac puncture and retina, liver and epididymal fat were collected for further analysis.

Experimental Methods

1. Serum glucose

Serum glucose was measured using a Genzyme Diagnostics™ (Charlottetown, PEI, Ref. # 220-32) colourimetric assay kit. The quinoneimine dye produced in the reaction between hydrogen peroxide, hydroxybenzoate and 4-aminoantipyrine, catalyzed by peroxidase was measured at its absorbance of 505 nm, and was assumed to be proportional to the amount of glucose in the sample. This test had a coefficient of variation (CV) of 3.1%.

2. Electroretinography (ERG)

Retinal function was determined by using full field electroretinogram (ERG) with the UTAS-4000 data system (LKC Technologies Inc., Gaithersburg, MD). To ensure maximum sensitivity, animals were dark adapted overnight before recording. To assess rod and cone function, mixed scotopic response and photopic response were used on the retina as previously published by our laboratory (Suh et al., 2009). ERG data were analyzed in a single eye per animal, chosen on the criteria of the greatest maximal scotopic a-wave amplitude. Schematic of full-field ERG apparatus is in Figure A-1.

Animals were prepared for bilateral ERG recording under dim red light. After application of anesthesia with an intraperitoneal injection of a mixture of ketamine (62.5 mg/kg) and xylazine (12.5 mg/kg), body temperature was maintained at 38°C with a heating pad and pupils were dilated using a drop of 1% tropicamide applied on the corneal surface, which also allowed electrical contact with the recording electrodes (gold wire loop). A pair of 25-gauge platinum needles, inserted subcutaneously behind each eye, served as reference electrodes, meanwhile a third 25-gauge platinum needle was placed behind the neck as a ground electrode.

2.1. Dark-adapted ERG

Dark-adapted, rod-driven intensity responses were generated by presenting a series of flash stimuli with increasing intensity (Suh et al., 2009). A single white flash (6500K, xenon bulb) was presented 3 to 5 times (to verify response consistency and obtain an average) at 14 increasing steps of intensity ranging from -3.70 to 2.39 log sc cd·s/m^2 (logarithm of scotopic candela seconds/meter square) in luminance. Inter-stimulus intervals were increased from 10 sec up to 2 min at the greatest stimulus intensity to allow maximal rod photo-bleaching recovery between flashes. For all recordings, a-wave (representing photoreceptor activation) was measured as the difference between baseline at 0 sec and the lowest point of the negative a-wave trough. B-wave amplitude (representing, in part, post-synaptic activation of ON-bipolar cells in the inner retina) was measured as the difference between the a-wave trough and the apex of the positive-going b-wave. The summed amplitudes of oscillatory potentials (representing activation of inner retinal neurons including amacrine and ganglion cells) were calculated using commercial software (EMWin from LKC Technologies Inc., Gaithersburg, MD).

2.2. Light-adapted ERG

Under light adaptation at 30 cd/m^2 background light, cone-driven intensity response was assessed by presenting single flashes (6500K, 10 μs duration) at 10 increasing steps ranging from -1.22 to 2.86 log cd·s/m^2. As with dark-adapted responses, a total of 5 light-adapted responses were averaged for each light intensity. Amplitudes were calculated as described above for dark-adapted responses.

3. Retinal immunohistofluorescence and immunohistochemisty

Following terminations, eyes were enucleated. Four left eyes from each group were placed in 4% formaldehyde solution overnight, and subsequently processed in a series of

sucrose gradients (10%, 20% and 30% in distilled water) for cryoprotection. After removal of the cornea and lens from the eyecups, they were placed in optimal cutting temperature (OCT) medium, and frozen in liquid nitrogen to be stored at -80 °C until processing. Along a naso-temporal orientation, 20µm sections were cryosectioned and mounted onto microscope slides. Briefly, 27 sections per retina were then washed in PBS (pH 7.4), and placed in blocking solution (10% goat serum and 0.3% Triton X-100 in PBS) and reacted with primary antibodies in (1% goat serum and 0.3% Triton X-100 in PBS) overnight at 20 °C in a humidified chamber. Monoclonal mouse anti-fibrillary acidic protein (GFAP) (1:1000) was used to assess Müller cell reactivity (Covance Inc., Princeton, NJ). Slides were washed in PBS and reacted with secondary antibody goat-anti-mouse-Alexa594 (Molecular Probes Inc, Eugene, OR) in 10% blocking solution for one hr in a humidified chamber before final PBS washes. A second alternate series of sections were stained with tomato lectin (Lycopersicon Esculentum) Dylight594 (1:100) to assess vasculature (Vector Labs, Burlingame, CA). Following staining either with GFAP or tomato lectin, three drops of ProLong® Gold antifade reagent (Molecular Probes Inc., Eugene, OR) containing nuclear stain 4',6-diamidino-2-phenylindole (DAPI) were applied to the slides, which were then cover-slipped to seal overnight. Once dry, sections were visualized and photographed using a Leica DM6000B brightfield microscope at 10x and 20x objectives. Images were projections of z-stacks of 5-13 slices of 1µm. Images were compiled using Adobe Photoshop CS2 software version 9.0.2 without adjusting for brightness or contrast (Adobe, San Jose, CA).

4. Carrot powder carotenoid composition & stability measurement

4.1. Chemicals, materials and preparation of standard stock solutions

Carotenoids, all-*trans*-β-carotene and *trans*-β-apo-8′-carotenal, and retinoids, all-*trans*-retinol, all-*trans*-retinal, retinoic acid, retinyl palmitate and retinyl acetate were

purchased from Sigma-Aldrich (Oakville, ON). Lutein, zeaxanthin and α-carotene for use as standards were generously donated by DSM Nutritional Products (Basel, Switzerland). Assay properties for all compounds were ≥95.0% (HPLC). Chemical structures are shown in Fig. 1-1 and Fig. 1-2. LC-MS grade acetonitrile, water, methanol and methyl t-butyl ether were obtained from Sigma-Aldrich (Oakville, ON). To minimize risk of compound degradation, standards and working solutions were prepared same day as extractions were initiated by appropriate dilution of the concentrated standard stock solutions with additional acetone. All stock solutions were prepared in HPLC grade acetone:chloroform (1:1) (Sigma-Aldrich, Ontario, Canada) and stored at -80 °C.

4.2. Sample preparation:

Carrot powder and diet samples

All sample preparation was performed under dim red light. The 4 compounds examined in the carrot powder, all-*trans*-β-carotene, α-carotene, zeaxanthin and lutein, were extracted with slight modification to the Wang and colleagues (2010) method. In brief, 100 mg of carrot powder or 100 mg of diet were extracted in triplicate with a mixture of 3 mL of chloroform:methanol (2:1, vol/vol), 2 mL of hexane, and 100uL of distilled water. Internal standard *trans*-β-Apo-8′-carotenal (8μg), a known metabolite in mammalian tissue, but not plants, was added to each sample, to determine the recovery threshold and help adjust for any loss of sample during extraction handling and procedures. Each sample mixture was vortexed, sonicated for 15 min. (Branson 3510, Danbury, CT) and then centrifuged at 2021 × g for 20 min. (Allegra 6R, Beckman Cloutier, Fullerton, CA). The upper organic phase was removed. This extraction was repeated twice more. Organic phases were pooled and evaporated to dryness under a nitrogen gas. The residue was redissolved in 175μl of chilled acetone, and 50 μl of the mixture was transfered to 300μl of chilled acetone to make the injection mixture. 10μl of

the injection mixture was injected into the column. Carrot powder samples yielded a 34.7-46% recovery for carotenoids. Triplicates were run and had intra-assay CVs of 13%, 18.5% and 11.5% for α-carotene, β-carotene and lutein, respectively.

Animal tissues

The extraction of retinoids and carotenoids from liver, serum and retina tissues was based on a modified method of Veda & Srinivasan (2011). Two hundred mg of liver homogenate, 500µl serum and 1 retina respectively were homogenized in a mixture containing 0.9% NaCl, 0.01% butylated hydroxytoulene (BHT) and retinyl acetate internal standard (Qiagen, Valencia, CA) for approximately 2 min. A 5mL mixture of chloroform:methanol (2:1, vol/vol) was then added to each aliquot, vortexed and centrifuged at 2021 × g for 15 min. The lower chloroform:methanol layer was evaporated to dryness under a nitrogen gas and transferred with two rinses to subsequent GC vial with insert using chloroform:methanol (2:1, vol/vol). Serum and retina were redissolved in 100µl and 50µl acetone, respectively. Liver samples were redissolved in 150ul after being extracted, and analyzed in triplicate.

Chromatographic conditions

Chromatography was utilized to quantify carotenoid pigments as previous spectrophotometric quantification has been reported to underestimate carotenoid status by as much as 50% when compared with HPLC (Fraser et al., 2000). The chromatography was performed on a Waters Acquity UPLC system coupled with photodiode array detection (UPLC-DAD), equipped with Acquity console software and MassLynx 4.1 (Waters Corp., Milford, MA). The column oven and autosampler temperatures were maintained at 25°C and 4°C, respectively. The autosampler was equipped with a door covering to minimize sample exposure to light. Carotenoids and retinoids were separated

on a C30 Carotenoid (3μm; 4.6 cm x 250 mm, Waters Ltd, Lachine, QC) reverse-phase column, with a gradient mobile phase at 0.4 mL/min (carotenoids) or 0.3mL/min (retinoids) consisting of methanol, methyl t-butyl ether and HPLC grade water (81:15:4, by vol, solvent A) and methanol, methyl t-butyl ether and HPLC grade water (6:90:4, by vol, solvent B).

Carotenoids

Carotenoid run conditions were as follows: Initial, 80% A, 20% B held until 15 min; gradient to 60% A, 40% B from 15 min to 23 min; isocratic hold 60% A, 40% B 23 min to 39 min; re-equilibration to initial composition of 80% A, 20% B from 39 min to 46 min. The total run time was 46 min. Detection wavelength was set at 420-460 nm for all standards and carotenoid compounds. An additional analogue detection was set at 326 nm to track the internal standard retinyl acetate in liver, serum and retina samples.

Retinoids

Retinoid run conditions were as follows: Initial, 100% A held until 11 min; gradient to 50% A, 50% B from 11 min to 18 min; isocratic hold of 50% A, 50% B from 18 min to 36 min; final re-equilibration to initial composition of 100% A from 36 min to 44 min with a hold at 100% A for 2 min. Total run time was 46 min. Detection wavelength was set at 320-385 nm for all standards and retinoid compounds.

Identification of retinoids and carotenoids was determined by comparing retention times and visible spectra with corresponding standards. The quantification of carotenoids was based on a calibration curves with known amounts of the standards and duplicate injections. The reference peak area (RPA) was plotted against a quantity of each standard compound over the area of internal standard. The linear regression equation was then used to calculate the concentrations found within the tissue extracts as determined by the ratio

of the known compound and the internal standard retinyl acetate. Some examples are shown in Figure A-2. Linear regression values for α-carotene, β-carotene, lutein, zeaxanthin, all-*trans*-retinol, all-*trans*-retinal and retinyl palmitate were > 0.98. Table A-1 includes a listing of all quantification equations and their associated regression values. Intra- and inter-day reproducibility were tested by preparing samples in triplicate and quadruplicate at four different time points and analyzing them by UPLC. Intra-day stability was evaluated from the measurement performed on the same day, while inter-day stability was obtained by measurement on four different days with carrot powder extractions. Detection limit for α-carotene and β-carotene was 0.025μg/injection with a signal to noise ratio (S/N) of 106.4 and 291.8, respectively. Detection limit for lutein and zeaxanthin was 0.005μg/injection with S/N of 25.8 and 34.0, respectively. 0.05ug/injection retinyl palmitate and 0.0033μg/injection all-*trans*-retinol and all-*trans* retinal were the lowest calibrants run, all samples had levels detected well above these detection limits. Consequently, signal to noise was not an issue for carotenoid or retinoid standards or samples, as the injection concentrations were far greater in samples. Liver samples yielded a 38% and 50% recovery for carotenoids and retinoids, respectively. Triplicates were run and had intra-assay CVs of 6% and 7% for retionol and retinyl palmitate, respectively. Retinoid recovery based on internal standard for retina (49%) and serum (60%) were also obtained.

5. Enzyme-linked immunosorbent assay of vitamin A transporters

Serum RBP4 and TTR were analyzed using AdipoGen (Cat.#AG-45A-0012EK-KI01, San Diego, CA) and Life Science Inc. (Cat.#E90726Ra , Missouri City, TX) enzyme-linked immunosorbent assay (ELISA) kits, respectively. Following all protocols provided, lower limits of detection were 1.05ng/mL TTR and 60pg/mL RBP4. Duplicates were run and had intra-assay CVs of 7.3 and 2.2% for TTR and RBP4, respectively.

6. Western immunoblot analysis of vitamin A transporters

Liver (40mg) samples were covered with liquid nitrogen and ground with a pestle to a fine powder. Samples were sonicated in a sample buffer containing 0.2 M Tris-HCl (pH 6.8), 3% SDS, and 30% glycerol (Sonic dismembrator, Model 100, Thermo Fisher Scientific Inc., Ottawa, ON) and centrifuged for 20 min at 13000 rpm (18000 x g) at 4ºC. The supernatant protein was measured by Pierce bicinchoninic acid (BCA) kit (Thermo Scientific, Rockford, IL, Cat.#23227). Ten µg of protein denatured by heat and 10% bromophenol blue (BPB): β –mercaptoethanol (β-ME) (v/v) was run through sodium dodecyl sulfate polyacrylamide gel electrophoresis (15% separating gel) to separate individual proteins including RBP4 and TTR based on their molecular weights. Proteins separated by the gel matrix were subsequently transferred to a positively-charged nylon membrane (PVDF) (Cat.#88518, Thermo Scientific, Gormley, ON). The membranes were then submerged in bovine serum albumin tris buffered saline with tween (BSA-TBST) blocking non-specific binding sites and then probed with primary antibody, specific to RBP4 and TTR (Cat.#71661B, #50256, Novus Biologicals, Oakville, ON) or loading control non-phosphorylated MAPK (Cat.#2146S, Cell Signaling, Danvers, MA). Following washing, secondary antibody conjugated with horseradish peroxidase (HRP) (Cat.#2146S, Cell Signaling, Danvers, MA) was applied to bind to the primary antibody. In the last step, bands were enhanced by chemiluminescence (ECL) kit (Cat#RPN2132, #RPN2133, AmershamTM ECL Plus Western Blotting Detection System, GE Healthcare, Buckinghamshire, UK) and quantified through a detection reader. Using FluorChemQ with AlphaViewQ image acquisition and analysis software (Alpha Innotech, California, USA), each was exposed at various time points using movie mode scanning technology to determine optimal intensity. Each membrane was then stripped with Restore™ Thermo Scientific stripping buffer (Cat.#PI21059, ThermoFisher Scientific, Nepean, ON) and re-probed with the next set of antibodies. As several membranes were probed for the same

protein, all bands were standardized using the intensity control standard to account for differences in exposure intensity. The intensity control standard was comprised of equal parts (75ul) of protein lysate obtained from one animal from each treatment which was selected based on median blood glucose level. Trace quantity (TQ) of each protein of interest was divided by the TQ of the intensity control located on the corresponding membrane. Similarly, protein levels were determined by comparison of TQs of proteins bands divided TQs of loaded control bands on the corresponding membrane. (For detailed methodology of all corresponding steps, please refer to Appendix A).

7. Statistical analysis

The effects of diet and disease were analyzed by two-way analysis of variance (ANOVA) using SAS 9.2 (SAS Institute Inc., Toronto, ON). The significant effects of the treatment were defined by Duncan's multiple range test. When there was no interaction effect of the diet and disease identified, the ANOVA was re-tested for the main factor, which enabled us to test against the correct degree of freedom of error. Liver carotenoids were tested by Student's t-test. Probability ≤ 0.05 was considered to be statistically different. Significant effects of treatment for ERG components were analyzed using the quadratic growth model with PROC MIXED provided by SAS. For this test, 11 out of 15 light steps of scotopic and and 6 of 10 light steps were tested due to the visible amplitudes. ERG data expressed as mean ± standard error of the mean (SEM). All other data expressed as mean ± standard deviation (SD).

Chapter 4: RESULTS

1. Carrot powder effect on physical and metabolic parameters

1.1. Body weight & food intake

Increasing dietary intakes were seen through the animal growth period (Fig. 3-1). From week 1 to 4 of treatment, animals were not distinguishable by weight. At week 3 immediately prior to diabetes induction, animals had similar intakes to carrot-enrichment animals (N 27.3±0.7, NC 26.1 ± 4 vs. D 24.9±3.0, DC 18.2±2.0g per day). This trend remained similar throughout the study. At week 4, following diabetes induction, diabetic animals had lower weights, with similar intake among all animals (Table B-1, B-2). The effects of dietary treatment and diabetes on body weight are presented as weekly means across the course of treatment (Table B-1). In animals provided carrot enrichment body weight was significantly increased ($P<0.038$) in healthy animals. At termination, STZ-induced diabetic control (D, 340.7 ± 33.2g) and carrot fed (DC, 354.5 ± 35.7g) animals weighed significantly less than healthy control (N, 540 ± 18.2g) and carrot fed (NC, 582.1 ± 47.3g) animals suggesting a loss in growth progression (Fig. 3-1; $P<0.0001$).

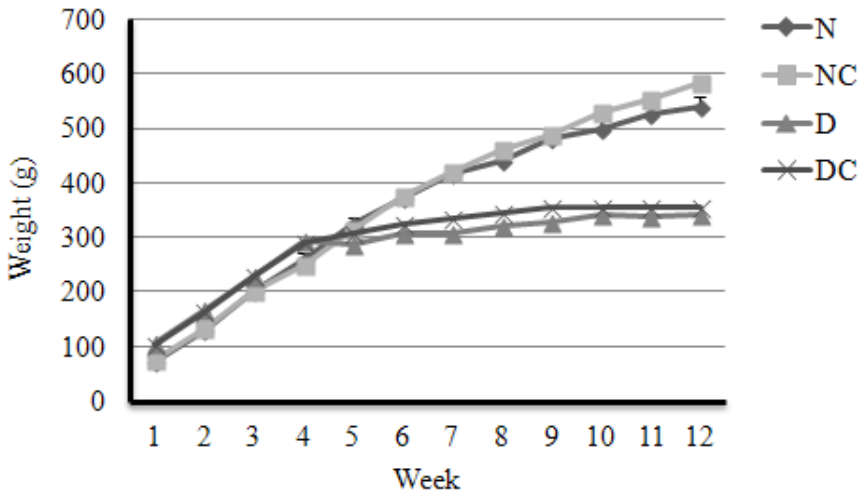

Fig 3-1: Carrot powder on body weights in health and Type 1 diabetes. Data expressed as mean ± SD (n=7-8 rats/group). Significant effects of diet (P=0.04) and disease (P<0.0001) at the end point were identified by two-way analysis of variance with a Duncan's multiple range test. N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment. See Table B-1 for tabulated weekly value.

1.2. Blood glucose and indicators of diabetes

Persistence of diabetes was confirmed by endpoint elevated fasted blood glucose. High fasting serum glucose at week 12 was indicative of diabetes in all D and DC animals (P<0.0001) in the range of 24.7 mmol/L to 40.58 mmol/L (Fig. 3-2). Diabetic animals receiving carrot diet had a reduced glucose concentration (15%) compared with diabetic controls suggesting some level of protective effect of carrot diet (diet and disease interaction P<0.04). Metabolic cage data confirmed polydipsia, polyuria, and polyphagia in diabetic animals (P<0.0001, P<0.0001, and P<0.0004, respectively) and has been summarized in Table B-3. Notably, fecal output was also significantly greater (62-65% more) in all diabetic animals. Carrot powder supplementation significantly decreased urinary output (33%) in the diabetic group. Together with glucose reduction, carrot-enrichment may have properties to improve diabetic parameters.

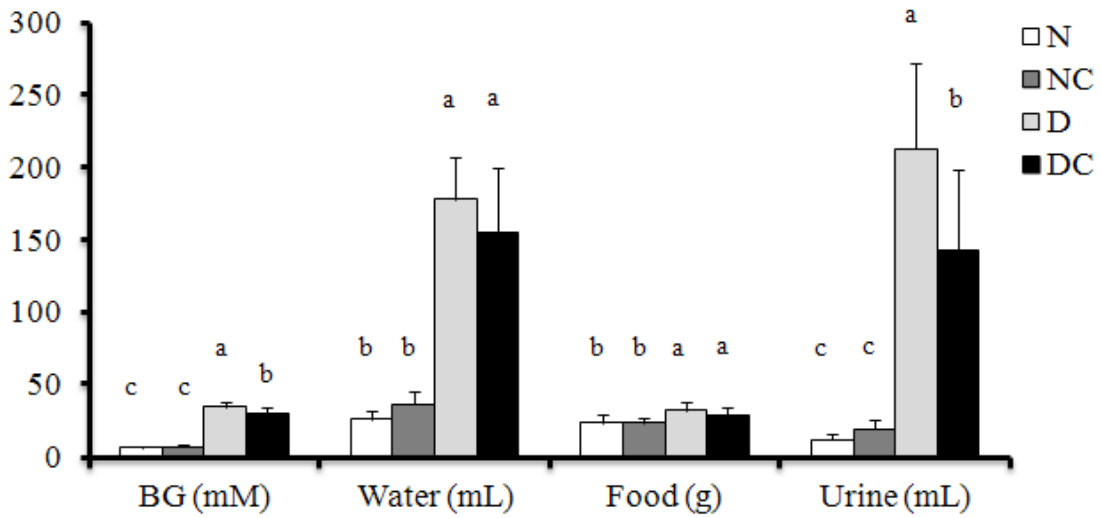

Figure 3-2: Carrot powder on metabolic cage parameters and endpoint blood glucose in health and Type 1 diabetes. Data expressed as mean ± SD (n=7-8 rats/group; exception BG: (N) n=3). Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test. Different superscripts within a parameter indicate statistical difference. Significant diet effects: urine ($P<0.03$). Significant disease effects: BG ($P<0.0001$), water ($P<0.0001$), food ($P<0.0004$), urine ($p<0.0001$). Significant interaction effects: BG ($P=0.041$), urine ($P<0.01$). BG, blood glucose; N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment. See Figure B-3 for tabulated values.

Table 3-2. Carrot powder on tissue weights in health and Type 1 diabetes

	N	NC	D	DC	Diet (P<)	Disease (P<)	D*D (P<)
Weight (g)	540 ± 18.3^{b}	582.1 ± 47.3^{a}	340.7 ± 33.2^{c}	354.9 ± 35.7^{c}	0.04	<0.0001	NS
Liver (g)	13.5 ± 6.1^{b}	17.9 ± 1.9^{a}	15.6 ± 2.4^{b}	20 ± 3.5^{a}	0.002	NS	NS
Adipose (g)	3.8 ± 1.1^{b}	5.5 ± 1.9^{a}	2.1 ± 0.4^{c}	1.9 ± 1.0^{c}	NS	<0.0001	0.032
Tissue weight relative to body weight (per 100g)							
Liver (g)	2.92 ± 0.21^{c}	3.08 ± 0.24^{c}	4.57 ± 0.54^{b}	5.66 ± 0.95^{a}	0.01	<0.0001	0.05
Adipose (g)	0.7 ± 0.21^{b}	0.95 ± 0.28^{a}	0.63 ± 0.13^{b}	0.53 ± 0.21^{b}	NS	0.005	0.033

All data expressed as ± SD (n=7-8 rats/group). Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test. Adipose represents epididymal fat.

1.3. Organ weights

Liver and epidydimal weights (g/ 100g body weight) were measured to determine the effects of carrot diet and diabetes (Table 3-2, Fig. 3-3). The weights for liver were significantly affected by carrot diet ($P<0.01$) and diabetes ($P<0.0001$) with interaction ($P<0.05$). Healthy rats fed control and carrot enriched diet had comparable liver weights (2.92 ± 0.21, 3.08 ± 0.24, respectively). Diabetic rats had 27-41% larger liver weights than their lean counterparts (4.57 ± 0.54, 5.66 ± 0.95, respectively). Diabetic animals fed the carrot diet (DC) had the largest liver size in proportion to body weight. Similar liver size trends have been observed in other studies of diabetes and vitamin A status (Basu and Basualdo, 1997; van Vliet et al., 1996). Epididymal adipose tissue was significantly reduced in diabetes ($P<0.005$) when compared to healthy animals. Healthy animals fed carrot diet exhibited the largest proportion of epididymal adipose tissue (Table 3-2).

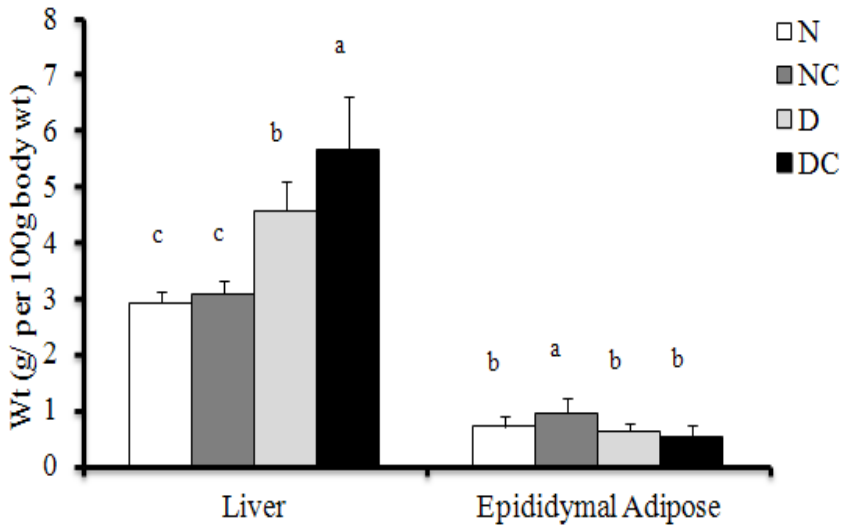

Figure 3-3: Carrot powder on liver and epididymal adipose weight in health and Type 1 diabetes. Data expressed as mean ± SD (n=6-8 rats/group). Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test. Different superscripts within a parameter indicate statistical difference. Significant diet effects: liver ($P<0.01$). Significant disease effects: liver ($P<0.0001$), epididymal adipose ($P<0.005$). Significant interaction effects: liver ($P<0.05$), epididymal adipose ($P=0.033$). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment. See Figure 3-2 for tabulated values.

2. Carrot powder effects on retina function

Rod and cone function of the retina were measured to determine the effects of carrot enrichment diet and diabetes using ERG recording.

2.1. Carrot powder on dark-adapted responses

Both carrot diet and diabetes significantly affected rod-driven cell function. Under dark-adapted conditions, a typical a-wave traces of healthy and diabetic animals are shown in Fig. 3-4A, B. Diabetic animals had significantly ($P<0.0003$) lower rod-driven photoreceptor (a-wave, Fig 3-4C) and bipolar cell (b-wave, Fig 3-4D; $P<0.01$) amplitudes compared to the healthy animals. When animals were provided carrot enrichment, both a-wave and b-wave amplitudes appeared higher in healthy animals, and lower in diabetic

groups (D and DC), with only b-wave reduction being significant (P<0.02) suggesting some impaired processing in the retina with diabetes (Fig. 3-4). This effect was most apparent at the highest stimulus strengths (elicited by 2.0 log cd· s/m^2). Rod driven a-wave and b-wave implicit times were also delayed in the diabetic animals provided carrot enrichment compared to all other groups, suggesting photoreceptor dysfunction, not reaching statistical significance (Fig. B-1). Reduced rod driven oscillatory potentials, an indication of diabetic retinopathy, were observed at increasing flash intensities in diabetic animals compared to the healthy animals (Fig. 3-4; P<0.005) with diet and disease interaction (P<0.04). This is consistent with human and streptozotocin-induced murine models of diabetic retinopathy which exhibited a reduction in oscillatory potentials (Shirao et al., 1998).

2.2. Carrot powder on light-adapted conditions

Although diagrammatical data trends suggest that carrot diet and diabetes affected cone-driven cell function, only a dietary effect reached statistical significance (P<0.01). In light adapted conditions, typical b-wave traces of healthy and diabetic animals are shown in Fig. 3-5A, B. When animals were provided carrot enrichment, b-wave amplitudes were higher in healthy animals, but lowest with diabetic carrot enrichment, indicating impaired processing in the retina with diabetes (Fig 3-5C; P<0.008). Cone driven bipolar cell b-wave responses appeared most reduced at all intensities in the diabetic carrot enrichement group (DC) compared to healthy animals (P=NS). B-wave implicit times were most affected by diabetes (D and DC) based on a two-way analysis of variance of maximal amplitudes (Fig. B-1; P<0.0004). Cone driven oscillatory potentials, an indication of diabetic retinopathy, were not statistically significant at increasing flash intensities in diabetic animals compared to the healthy animals (Fig. 3-5D).

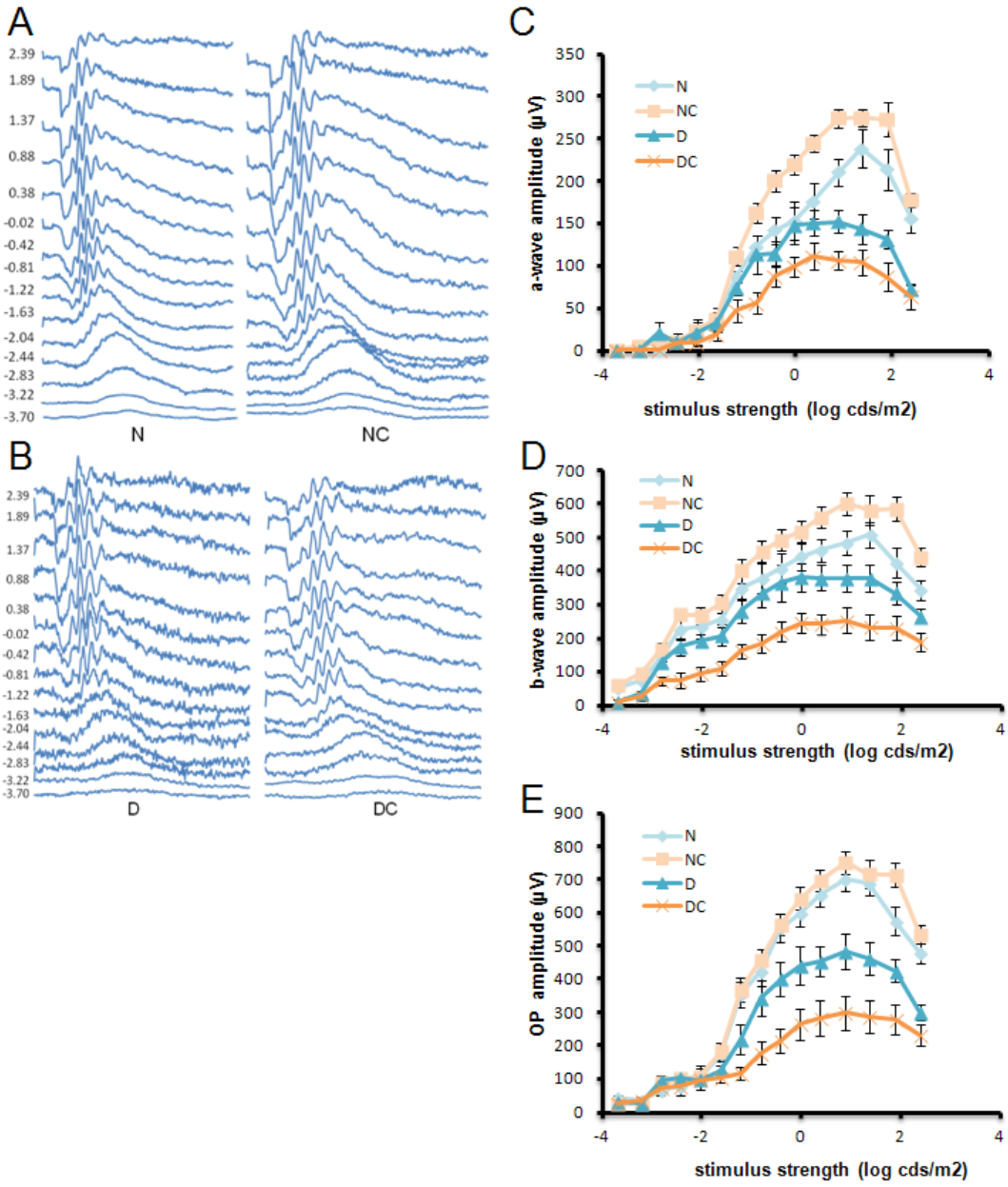

Figure 3-4: Carrot powder on ERG dark adapted responses in health and Type 1 diabetes. Data expressed as mean ± SD (n=6-8 rats/group). Significant effects of diet and disease were identified by using quadratic growth model with PROC MIXED provided by SAS. Representative ERG traces: (A) ERG for N compared with NC; (B) ERG for D compared with DC. Scotopic responses: (C) A-wave amplitude; (D) B-wave amplitude; (E) Sum oscillatory potential (OP) amplitude. Significant diet effects: a-wave (NS), b-wave ($P<0.02$), sum OP (NS). Significant disease effects: a-wave ($P<0.0003$), b-wave ($P<0.01$), OP ($P<0.005$). Significant interaction effects: a-wave (NS), b-wave ($P<0.05$), sum OP ($P<0.04$). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

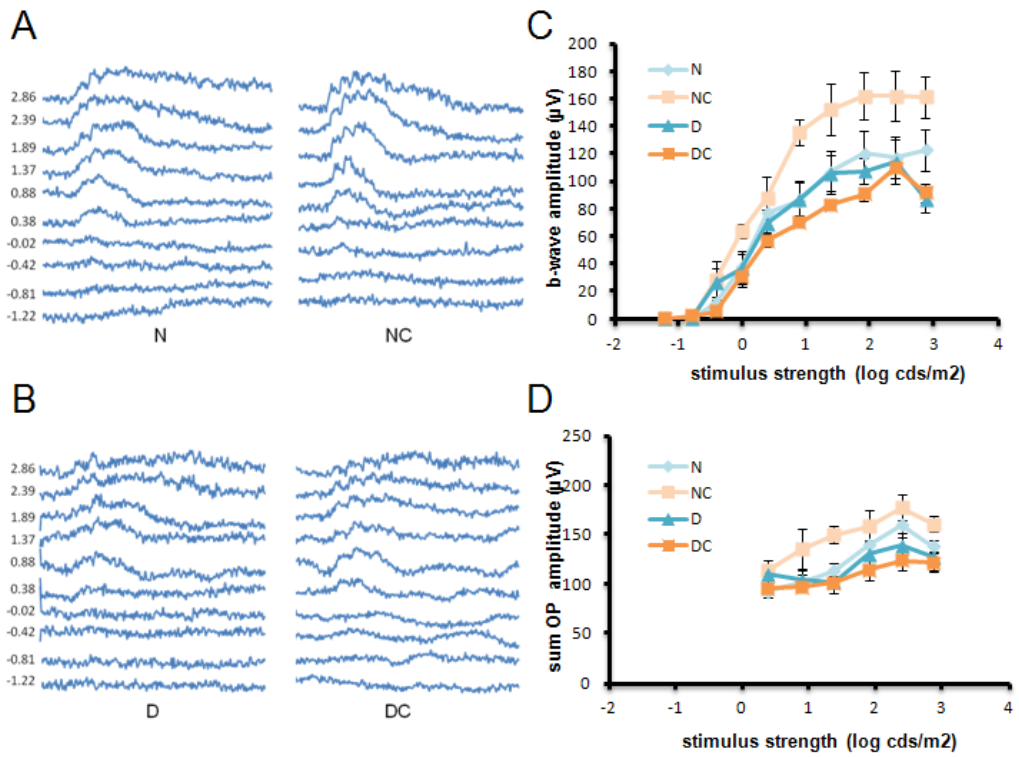

Figure 3-5: Carrot powder on ERG light adapted responses in health and Type 1 diabetes. Data expressed as mean ± SD (n=6-8 rats/group). Significant effects of diet and disease were identified using quadratic growth model with PROC MIXED provided by SAS. Representative ERG traces: (A) ERG for normal control (N) compared with normal carrot enriched (NC); (B) ERG for diabetic control (D) compared with diabetic carrot enriched (DC). Photopic responses: (C) B-wave amplitude; (D) Sum oscillatory potentials (OP). Significant diet effects: b-wave ($P<0.01$), sum OP (NS). Significant disease effects: b-wave (NS), sum OP (NS). Significant interaction effects: b-wave ($P<0.008$). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

3. Carrot powder effects on retina anatomy

3.1. Muller cell reactivity (GFAP)

At study endpoint, the expression of retinal glial fibrillary acidic protein (GFAP), a marker of Müller cell reactivity and retinal injury was examined in the ganglion cell layer of the inner retina both centrally and peripherally (Fig. 3-7). GFAP filaments were expressed normally in the inner half of the retinal Müller cells and their endfeet indicating

early stage of diabetic retinopathy. GFAP would be expected to be significantly upregulated throughout the cell with greater disease severity (Guerin et al., 1990).

3.2. Retina morphology (DAPI)

Retina morphology was assessed using 4,6,diamidino-2-phenylindole (DAPI) nuclear stain of the same sections. Outer nuclear layer cell row counts (ONL, 11-13 cell rows) were similar in all groups within both the central and peripheral regions of the retina. Similar nuclear cell rows among all treatments were also found in the inner nuclear layer (INL) containing closely packed bipolar, horizontal and amacrine cells (Fig. 3-6). No significant photoreceptor losses, indicative of advanced diabetic retinopathy, were observed in either the central or peripheral retina between carrot powder supplementation or diabetes, and is indicative of early stage diabetic retinopathy, characterized by functional changes (as assessed by the electroretinogram), without structural changes (Phipps et al., 2006).

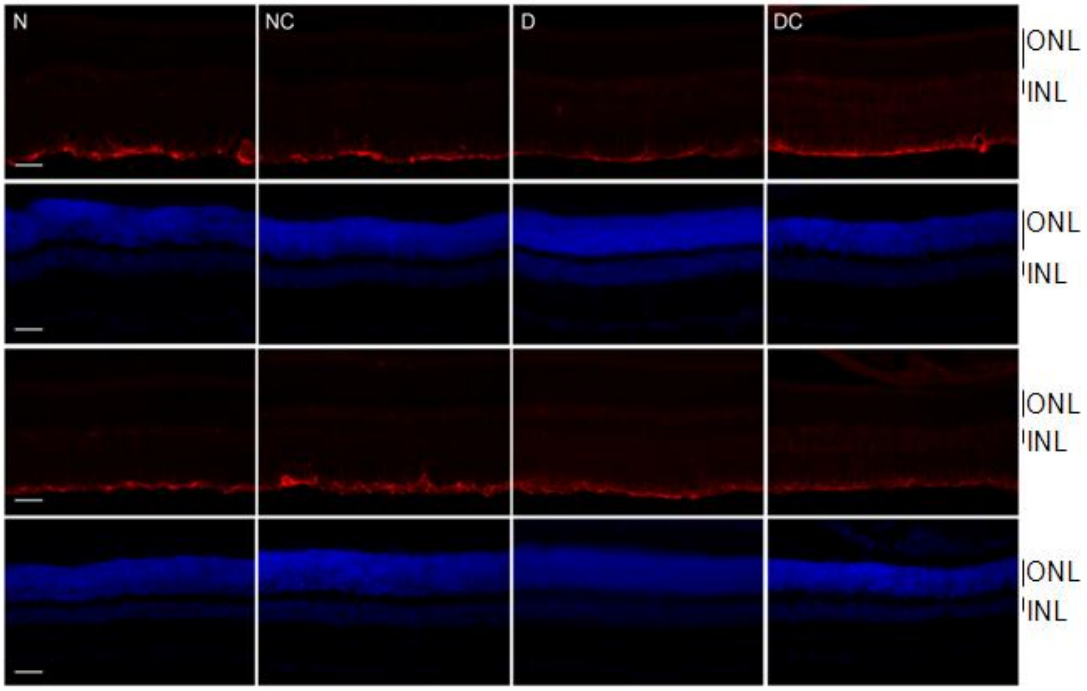

Figure 3-6: Carrot powder effect on GFAP Müller cell reactivity staining (red) and DAPI nuclear visualization (blue) in health and Type 1 diabetes. Representative central (1st & 2nd row) and peripheral (3rd & 4th row) retina, 20X objective. ONL, outer nuclear layer; INL inner nuclear layer. N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

3.3. Lycopersicon esculentum vascular stain (tomato lectin)

Tomato lectin vascular staining for blood vessels and microglial cells is captured in Fig. 3-7 at retina mid-point in each treatment group. There were comparable levels of retinal vasculature, which were overall, representative of healthy normal retina across all groups at study endpoint. Tomato lectin staining of the diabetic groups (D and DC) demonstrated early indices diabetic retinal angiogenesis in some retina sections by the presence of vessel strictures and tortuosity in the inner plexiform and inner nuclear layers. Early and infrequent development of vascular angiogenesis in the ONL was also apparent in a small number of sections from the D and DC groups and is represented in Figure 3-7 by an example of a DC retina.

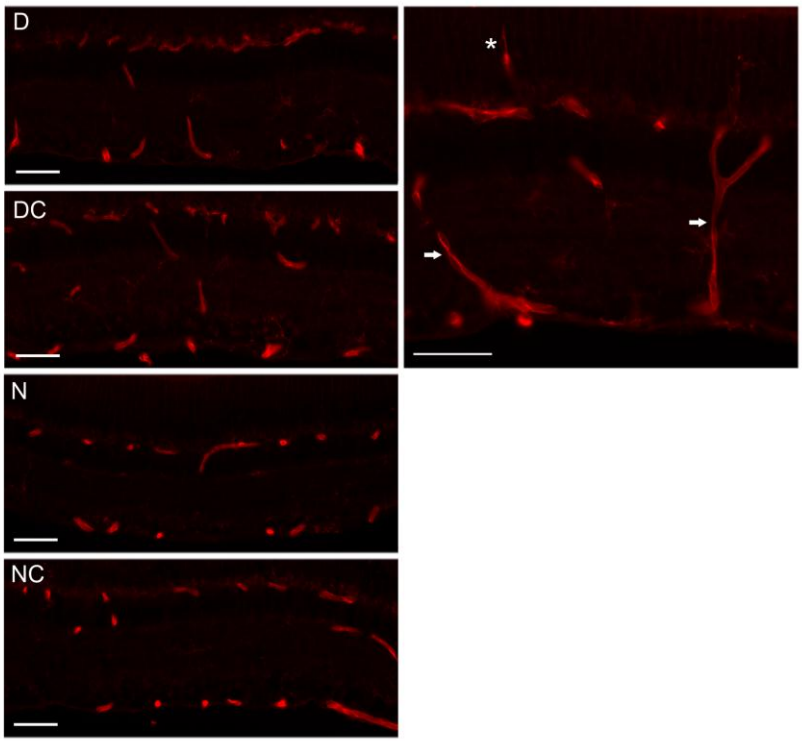

Figure 3-7: Carrot powder effect on tomato lectin vascular staining in health and Type 1 diabetes. Captured at retina mid-point, 10X objective. Example of indices of early stage diabetic retinopathy in D & DC groups (left). Enlarged view of diabetic vessel structures; *indicates vascular angiogenesis in the ONL, →indicates vessel tortuosity and stricture. NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

4. Carrot powder effects on retinoid and carotenoid status: serum, liver, retina

Retinoid and carotenoid levels were measured in the liver, serum and retina to examine if carrot powder influences the bioavailability of carotenoids and retinoids in health and Type 1 diabetes.

4.1. Carrot powder effects on liver vitamin A and carotenoids

Liver retinoid concentrations (µg per g of liver tissue) were measured to determine the effects of carrot diet and diabetes (Fig. 3-8). The levels of retinol ($P<0.04$) and retinyl palmitate ($P<0.0001$) in the liver were significantly increased by feeding carrot enriched diet in both healthy and diabetic animals. Healthy rats with carrot enrichement had the highest liver retinol and retinyl palmitate reserves (43.13 ± 7.44, 743.45 ± 160.16 µg/g

liver, respectively). As a result, healthy animals provided carrot enrichment had levels of retinol increased by 1.3 times and retinyl palmitate by 3.4 times compared to the healthy controls without carrot enrichment. However, carrot enrichment in diabetes led to lower levels than in their healthy carrot-enriched counterparts (27.98 ± 10.17, 383.19 ± 100.51 μg/g liver, respectively). Diabetes significantly reduced the levels of retinol ($P<0.003$) and retinal palmitate ($P<0.0005$). Retinol was significantly decreased in groups of diabetic animals by a percentage change of 36%, 35% respectively, for control and carrot enriched groups when compared with their healthy counterparts. Similarly, a decrease was also identified in retinyl palmitate with a percentage difference of 23%, 48% respectively, for diabetic control and carrot enriched groups, suggesting impaired conversion and storage or increased mobilization in diabetes. There was more pronounced levels of retinyl palmitate in the carrot enriched groups, with a significant diet and disease interaction ($P<0.0055$).

Alpha and β-carotene were only evident in chromatograms from the carrot enrichment diet animals (NC and DC) (Fig. 3-8). Lutein and zeaxanthin were not detected in any liver extracts. Examples of retinoid and carotenoid chromatograms from diet and animals in each dietary treatment are shown in Fig. B2-B7. Levels of α and β-carotene ($P<0.006$) were 68% and 86% higher in the diabetic animals when compared with their healthy counterparts, although there was inter-animal variability.

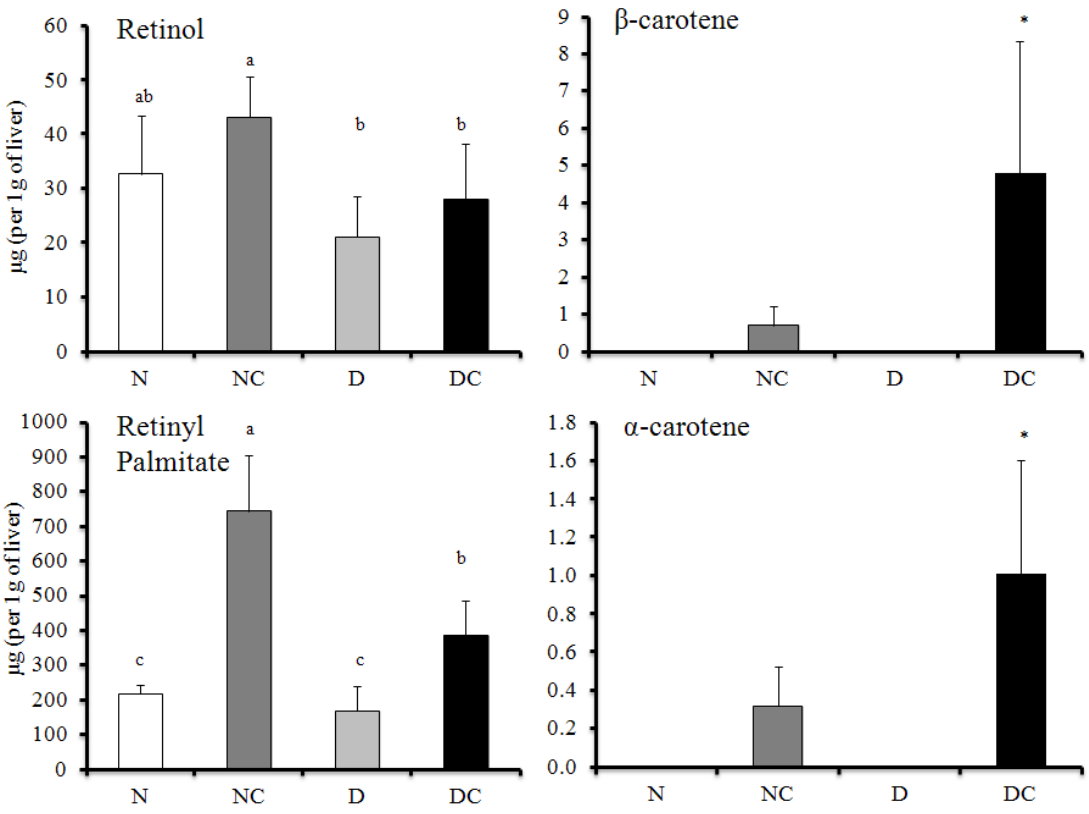

Figure 3-8: Carrot powder on liver retinoids and carotenoids in health and Type 1 diabetes. Expressed as ug/g of liver tissue. Data expressed as mean ± SD (n=7-8 rats/group, exception (N) n=3). Significant effects of diet and disease on retinoids were identified by two-way analysis of variance with Duncan's multiple range test. Different superscripts within a parameter indicate statistical difference. Significant diet effects: retinol ($P<0.04$), retinyl palmitate ($P<0.0001$). Significant disease effects: retinol ($P<0.003$), retinyl palmitate ($P<0.0005$). Significant interaction effects: retinyl palmitate ($P<0.0055$). Significant effect of diet on carotenoids were identified by Student's t-test ($P<0.006$). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

4.2. Serum vitamin A and carotenoids

Serum samples were analyzed for retinol, retinyl palmitate, β-carotene and α-carotene, however only retinol was detected in fasted serum. No significant differences were found in serum retinol levels among groups (Fig. 3-9). Animals fed the carrot diet had higher retinol than their counterpart healthy (13%) and diabetic (9%) animals but values did not reach significance ($P=0.09$).

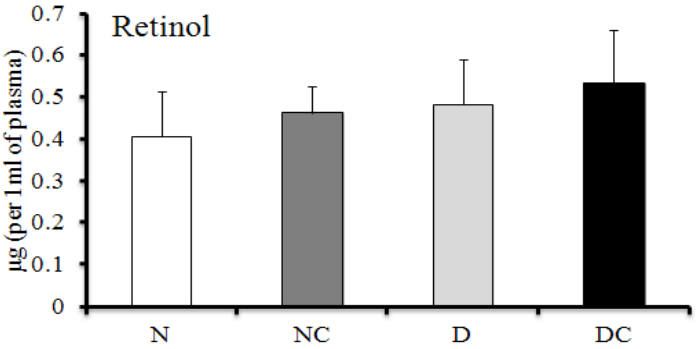

Figure 3-9: Carrot powder on serum retinol in health and Type 1 diabetes. Expressed as retinol contained in 1ml of serum. Data expressed as mean ± SD (n=7-8 rats/group; exception (N) n=3). No statistical differences were identified by two-way analysis of variance. N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

4.3. Retina vitamin A and carotenoids

Retinol and all-*trans*-retinal were detected in retina of all animals, but not α-carotene and β-carotene, lutein, and zeaxanthin. Although all-*trans*-retinal was used as a standard, we speculate that this peak might be the *cis*-isomer form of retinal, which could be co-eluted, as the animals were dark adapted during retina isolation. Retinol and retinal levels (0.26-0.40 ± 0.11, 2.34-3.66 ± 1.24 µg per retina, respectively) were not affected by diet or diabetes (Fig. 3-10).

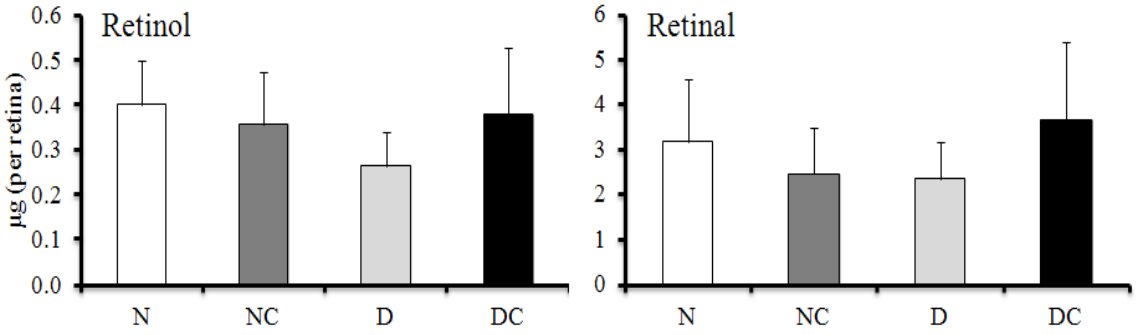

Figure 3-10: Carrot powder on retina retinoid content in normal and Type 1 diabetes. Expressed as total content of one retina. Data expressed as mean ± SD (n=7-8 retinas/group; exception (N) n=5). No statistical differences were identified by two-way analysis of variance. N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

5. Retinol transporters in serum and liver (RBP, TTR)

Retinol transporters were measured in the serum and liver to examine if they are influenced by carrot powder diet and Type 1 diabetes.

5.1. Serum

The level of RBP4 was significantly increased by diet ($P<0.0001$), disease ($P<0.0001$) and diet and disease interactions ($P<0.001$) measured by the ELISA assay (Fig. 3-11). Healthy animals that were not provided carrot enrichment had the lowest levels of RBP4 in comparison to all other groups.

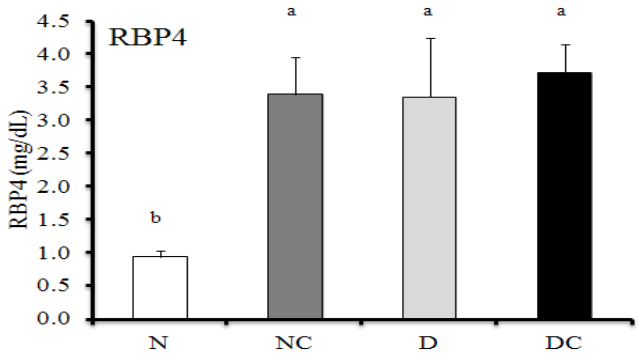

Figure 3-11. Carrot powder on serum retinol transport protein levels in health and Type 1 diabetes. (A) RBP4. All data expressed as mean ± SD (n=7-8 rats/group; exception (N) n=3). Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test. Different superscripts indicate statistical difference. Significant diet effects ($P<0.0001$). Significant disease effects ($P<0.0001$). Significant interaction effects ($P<0.001$). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

5.2. Liver

The level of retinol binding protein 4 (RBP4) and transthyretin (TTR) were measured in the liver by Western blot analysis. The level of RBP4 was significantly ($P<0.0008$) increased in the diabetic animals compared to their healthy counterparts; no dietary effect was identified. RBP4 results are consistent with RBP4 upregulation in mice

with insulin resistance (Yang et al., 2005). TTR bands were shown in their trimeric, dimeric and monomeric forms at 42kDa, 27kDa and 16kDa, respectively. No dietary or disease effects were seen in TTR protein band levels at 27kDa and 16kDa (data not shown). There was significantly increased protein banding of TTR at 42kDa in both diabetic groups (D & DC) (P<0.0001) with interaction (P<0.03) (Fig. 3-12). There were no carrot enrichment induced differences in levels of either of the transporter proteins.

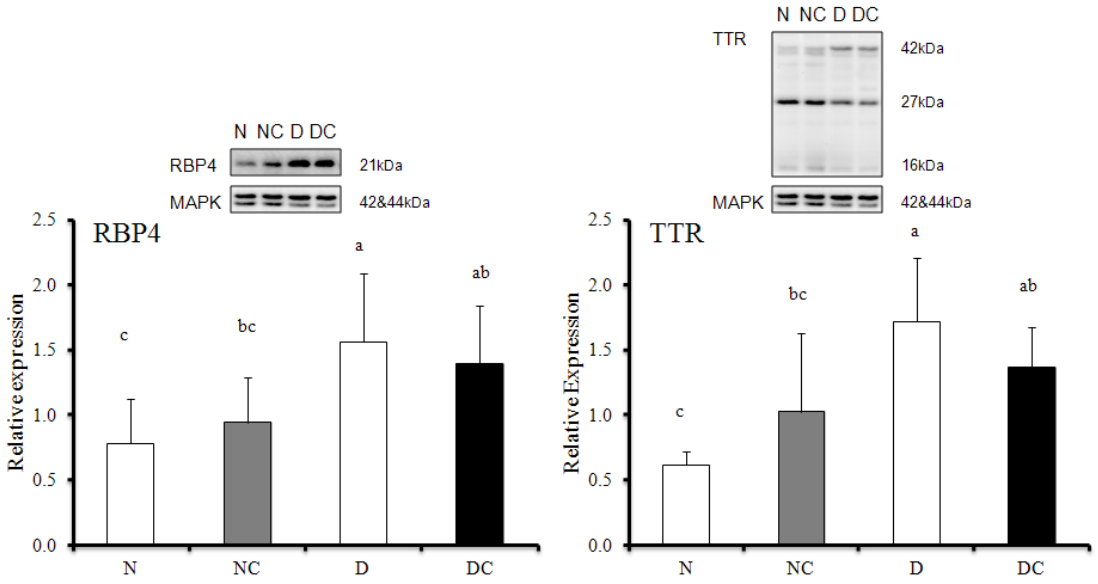

Figure 3-12. Carrot powder on liver retinol transport protein levels (L) RBP4 (R) TTR 42kDa in health and Type 1 diabetes. Transporters probed on the same membrane. All data expressed as mean ± SD (n=6-8 rats/group) in arbitrary units. All data were adjusted to control for differences in band intensities and loading. A representative Western blot is included on top of corresponding protein graphs. Protein levels were adjusted to control for differences in protein loading. Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test. Different superscripts within a parameter indicate statistical difference. Significant disease effects RBP4 (P<0.0008), TTR 42kDa (P<0.0001). Significant interaction effects TTR 42kDa (P<0.03). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

Chapter 5: DISCUSSION

1. Influence of carrot powder supplementation on diabetic parameters

1.1. Influence of carrot powder on food intake and body weight

Dietary intake for both treatments were similar, and correspond with a previous published study that used same amount of (15%, w/w) carrot powder supplementation to assess its therapeutic effects on cholesterol (Nicolle et al., 2003). Greater animal weight with β-carotene supplementation was seen in a study of ferrets provided β-carotene (3.2 mg per kg of body weight daily) for 6 months. The group of animals who were supplemented had weights that were 14% greater than that of controls (Murano et al., 2005). Our diabetic animals averaged a gain of only approximately 56g post-STZ injection in comparison to 310g seen in healthy animals. Consequently, diabetes in our study was characterized by a lack of growth, and weight loss, including epididymal adipose loss with increased duration of diabetes. One cross-sectional study of Type 1 diabetes patients with longer duration diabetes showed a 10–20% reduction of the fat mass after 7–10 years of disease onset (Rosenfalck et al., 2002). A previous longitudinal study found that in the poorest quartile of metabolic control (HbA_{1c} > 8.9%), there was significant reduction in lean body mass during the initial 3 years after diagnosis (Sinha et al., 1996). Future studies should examine the effect of carrot powder supplementation on body compositional changes occurring with diabetes to determine if the weight-protective effect correlated with sparing of lean mass, fat mass or if there was any organ weight involvement.

1.2 Influence of carrot powder on metabolic parameters

The present study found that carrot enriched diet improved fasting serum glucose and decreased urinary output by 33% in the diabetic animals. It also increased stool weight. Although not tested, better glucose handeling effects of carrot may be a result of the food matrix containing sugars, and/or the soluble fiber content of the carrot which

may provide a different response than semi-purified forms contained in the control diet.

2. Influence of carrot powder on retinal function

To date, this is the first study examining the effects of carrot powder on retinal function measured directly by ERG response. Healthy animals receiving carrot powder had significantly greater rod-driven bipolar cell (b-wave) amplitudes than all other groups suggesting improved inner retina visual function. Rod-driven photoreceptor cell (a-wave) amplitudes demonstrated a similar trend, without reaching statistical significance. These results may relate to a higher availability of pro-vitamin A carotenoids from carrot powder which may have increased the availability of vitamin A used for chromophore within the retina in healthy animals receiving carrot enrichment. However, retina levels of retinol and retinal were not significantly different among the groups.

In regards to retinal function measured electrophysiologically in vivo, at 9 weeks post STZ injection, male Wistar rats displayed the landmark functional retina defects (decreased ERG OP amplitudes), which confirmed early stage diabetic retinopathy, similar to reports in 6 weeks post-treatment in rats (Shirao and Kawasaki, 1998). In addition, delayed OP latencies have been reported at 2 weeks post-treatment, reinforcing that OP changes are an early phenotype of diabetic retinopathy. The diabetic rats also showed photoreceptor and inner retinal dysfunction. A similar finding has been reported 2 days following induction of diabetes (Phipps et al., 2004).

Unlike healthy animals, diabetic animals receiving carrot powder had further reduced rod-driven photoreceptor (a-wave), bipolar cell (b-wave) amplitudes than animals fed the control diet. A-wave implicit times in the carrot fed diabetic group held the greatest latency. As evidenced by these results, inner retina function was more affected, perhaps as a result of diabetic vascular abnormalities identified in the tomato lectin staining. This suggests that trends in implicit time latency of photoreceptors in the Type 1

diabetic condition, and subsequently rod and cone driven pathways were detrimentally impacted by the carrot powder enriched diet. In other words, where carrot powder supplementation proved beneficial in healthy rats, by improving b-wave light and dark-adapted amplitudes, it trended towards defects in diabetes. Similar levels of retinal and retinol in the retina among all the groups did not provide a clear association of retinoid involvement in this case. Since we provided high concentration of carrot, other components in the carrot, such as fiber source, may have played a role in the detrimental effects in diabetes.

As a general comparison, greater reduction in cone b-wave amplitude rather than rod b-wave in our animal model might be due to redundancy of rat cone pathway, rather than dietary treatment differences. Consequently, OPs were found to be most reliable indicator of diabetic retinopathy, with no significant dietary differences being noted. It has also been postulated that cone cells, obtaining their retinol from the Vitamin A cycle located in Müller cells, are exposed to higher levels of glucose in diabetes, which may account for increased apoptosis over rod photoreceptors (Owsley et al., 2006). In humans, diabetic retinopathy would first cause greater reduction in cone-driven a-wave, rather than rod-driven a-wave. However, recent comparison of murine models of age-related photoreceptor degeneration (Charng et al., 2011) suggest that cone-driven a-wave is more difficult to record in albino rats, such as those used in our study, due to their higher amount of rod photoreceptors compared to humans. Authors further found a reduction in a-wave, which corresponded to a loss in photoreceptors and outer segment shortening, suggesting that albino models may be less resistant to cone loss than pigmented models (Cano et al., 1986; Lai et al., 1978).

Anatomically, diabetic retinopathy is characterized by abnormal retinal vasculature, paired with glial (Müller cell) and neuronal cell changes (Mizutani et al.,

1998). In this study, diabetic animals regardless of dietary treatment showed no differences in injury marked by protein filaments measured by GFAP in Müller cells. The DAPI staining of nuclei further confirmed no significant alterations to the nuclei of the outer or inner nuclear layers. Similar findings have been reported for nuclear staining, with no notable differences in rod and cone photoreceptor density or structure at 12 weeks post-STZ diabetes induction, despite clear ERG changes (Yee et al., 2010). However, authors did identify significant labeling in Müller cell processes of STZ-induced rats at 12 weeks. It has been reported that Müller cells are affected in more severe human and experimental diabetes, which is accompanied by decreased glutamine synthetase, glutamate transporters and markers of inflammation (Gerhardinger et al., 2005). Consistent with retinopathy, our diabetic animals displayed the start of anomalies in retina vasculature, including sporadic presence of vessel strictures, vessel tortuosity and the early presentation of vascular angiogenesis in the ONL with tomato lectin stain visualization. Research into advanced stages of diabetic retinopathy report breakdown of the blood retinal barrier, vascular leakage, and pronounced neovascularization (Qazi et al., 2009). Provided this information, there still exists gaps in knowledge surrounding how neuroretinal lesions and microvessel histopathology are produced in diabetes. The discrepancy between functional and structural changes in our study may reflect the variable duration or severity of diabetic hyperglycemia reported in the literature. Reducion in ERG b-wave may correspond with inner retina neovascularization. Rhodopsin and cone opsin quantitave immunolabeling of the photoreceptors was not performed in our study; subsequently future research should examine the level of rhodopsin or cone opsin expression under similar conditions of dietary manipulation and hyperglycemia to examine the effect of vitamin A bioavailability on functional changes. When considered collectively, lack of anatomical damage, early signs of abnormal vasculature and reduction in inner retina function as assessed by the ERG recordings in

both diabetic groups suggest that both diabetic groups have the characteristics of early stage diabetic retinopathy, with ERG detecting early funcational changes.

Overall our findings may reflect a diabetes-induced change in vitamin A distribution or clearance within the retina for the visual cycle which causes ERG abnormality in STZ-induced rats before structural damage can occur at a greater severity than those that did not receive carrot enrichment. It may be conceivable then, that the damaging effect seen in diabetic animals receiving carrot enrichment may involve vitamin A substrate availability for the visual cycle, as UPLC method determined total vitamin A content of the retina, without the ability to discern location or distribution. Interphotoreceptor retinal binding protein (IRBP) may have been less efficient at transporting 11-*cis*-retinal (or clearing all-*trans*-retinal) to and from the rod outer segment in diabetes (Simo et al., 2010). Carrot powder enrichment in diabetes may have also contributed to an increase in asymmetric cleavage carotenoid metabolites in ocular tissues with uncharacterized effects (Bernstein et al., 2001; Khachik et al., 1997). Further work should assess visual cycle transporter and enzyme levels (such as IRBP or LRAT), and establish a dose-response between carrot powder supplementation and retinal function indices. Function of Na+/K+ ATPase activity in diabetes, should also be considered as it has also been seen as a mechanism by which photoreceptor amplitude is reduced in diabetic rats (Ottlecz et al., 1993). Additionally, in order to factor in applicability of human health, insulin injection should be assessed for its effect on study parameters, and to rule out any independent effect of hyperglycemia. As reported by Kowluru and colleagues, daily insulin (2 IU/d) can reverse the G-protein deficiency within the visual cycle induced by diabetes and may explain why some studies report normal sensitivity in rats given insulin, also implying that human patients with abnormal sensitivity have hyperglycemia or poor control of glycemia (Kowluru et al., 1992).

3. Influence of carrot powder on liver

3.1 Influence of carrot powder on liver size and vitamin levels

Since liver, and more specifically the perisinusoidal stellate cells, are the primary storage site and regulator of vitamin A homeostasis, we measured the liver size and retinoid and carotenoid levels to reflect effects of carrot enrichment. Liver stores 50-85% of a human's reserves in vitamin A (0.147-0.441 μmol/g) as retinyl esters in lipid droplets (Blaner et al., 2009; Goodman, 1984; Olson, 1984; Penniston and Tanumihardjo, 2006). These droplets exist in membrane bound and unbound forms (Wake, 1980). In our study, the diabetic animals had 27-41% larger livers than their lean counterparts, with carrot diet contributing to the largest liver sizes of all groups. It has been reported that humans have larger liver size due to chronically poor glycemic control and insulin resistance in Type 1 diabetes (Bulum et al., 2011; Murata et al., 2012), and hypervitaminosis A induced inflammation and fibrosis (Nollevaux et al., 2006). Whether carrot induced increased liver size has similar detrimental effect needs to be examined.

Notably, animals receiving carrot powder enrichment had larger livers paired with detectable α-carotene and β-carotene reserves, which were not detectable in the control diet-fed animals. The accumulation of α-carotene and β-carotene in diabetes was 68%, and 86% greater respectively, than in the healthy carrot enriched animals. Carrot enrichment also corresponded to significantly greater accumulation of retinol and its ester storage form retinyl palmitate, but accumulation was much lower in diabetes. Based on these results, carrot powder supplementation had the greatest impact on increasing liver size in diabetic rats. Similar increased liver size has been observed in studies of diabetes and vitamin A status (Basu and Basualdo, 1997) and vitamin A and carotenoid supplementation in healthy Sprague-Dawley rats (van Vliet et al., 1996). Conversely, in healthy and diabetic Wistar rats only a trend of increased liver size with diabetes, and not vitamin A supplementation was seen (Tuitoek et al., 1996c).

Finding higher storage of carotenoids with lower retinoids in the liver in our study suggests there may be impairment of α-carotene and β-carotene conversion to retinol and retinyl palmitate within the liver during diabetes. In supplementation studies of healthy animals, it appears that cleavage and conversion activity in the liver is greater, as there are lower levels of carotenoids, and higher amounts of vitamin A. In healthy state, β-carotene absorption and cleavage is regulated at the intestinal level, with reduced activity, protecting the organism against an undesirable retinoid accumulation. This system may not work well in diabetes, however it is only speculative as carotenoid and lipid metabolism enzymes were not directly measured in this study. Findings may also relate to the loss of adipose tissue, which is another large storage site of carotenoids. Recently a study following patients 6 months after gastric bypass found that weight loss was accompanied by a significant increase in serum β-carotene (Boesing et al., 2010). Baseline blood levels of β-carotene were lower in participants than in controls, suggesting that the weight loss, more specifically adipose loss, may have liberated β-carotene from adipose reserves, in turn causing the β-carotene to be re-distributed in the serum, and among other organs.

Mounting evidence suggests an association between Type 1 diabetes and the development of non-alcoholic fatty liver (NAFLD) in Type 1 diabetes (Leeds et al., 2009; Targher et al., 2010; West et al., 2010). Provitamin-A carotenoids and retinoids may also be involved in the change in liver size, and potential NAFLD present in our animals. Due to the light sensitive nature of the retinal work, all tissue collection was completed in the dark, and we were unable to visually assess for focally evident liver steatosis with diabetes or carrot powder enrichment. The next step of the project will be to test for the existence of steatosis. In healthy ferrets, it has been reported that a daily dose of β-carotene (3.2 mg /kg body weight/day) for 6 months may contribute to adipocyte secretion of vasculogenic adipokines which in turn increase lipid accumulation and

progression of enlarged livers with steatosis, as was seen in half of the animals (Murano et al., 2005). Body weight was 14% higher in animals receiving the higher dose of β-carotene than in controls, liver enlargement, but not weight, was reported. The central part of the lobules with portal areas showed infiltration of mononuclear cells. Large vacuoles likely representing lipid droplets inside the hepatocytes with focal distribution were also present in the supplemented animals (Murano et al., 2005).

3.2. Influence of carrot powder on liver vitamin A transporters

As proteins are required in the cellular uptake and transportation of carotenoids, it is pertinent to consider the consequences modulation of protein expression and activity on carotenoid blood and tissue concentrations in a diabetic state. The primary route of vitamin A delivery from the liver to the retina requires retinol bound to retinol binding protein (RBP) and transthyretin (TTR) (Dixon and Goodman, 1987). TTR is synthesized predominantly by the hepatic parenchymal cells and behaves like a tetramer, binding to RBP4 and thyroxin in the plasma (Peterson and Rask, 1971). In fact, both TTR and RBP4 proteins are chiefly synthesized in the liver, with lesser amounts being produced by adipocytes. Expression of these transporters is known to be regulated by liver vitamin A status. In our study, both TTR (42kDA, trimeric form) and RPB4 are significantly upregulated in diabetes in comparison to the non-diabetic animals, but expression was not affected by carrot enrichment in disease. Upregulated expression of RBP has been reported in mice with insulin resistance (Yang et al., 2005). This is the first study to examine the different forms and levels of TTR (monomer, dimer, trimer, and tetramer) in the liver in Type 1 diabetes by Western blot. Our model of early diabetic retinopathy does not correspond the observations reported in vitamin A deficiency (decreased RBP) and states of infection, inflammation and the acute phase response (decreased TTR and RBP) seen in humans (Rosales et al., 1996) indicating that deficiency and acute inflammation are not the primary triggers of metabolic derangement in Type 1 diabetes.

Previous studies conducted by the Basu research group (Basu et al., 1989; Tuitoek et al., 1996a) found a reduction in RBP and TTR in Type 1 diabetic rats and humans, however results may be less comparable due to the severity of diabetes, and different experimental techniques measuring transport proteins (radioimmunoassay versus Western blotting). Additionally, unlike the animals studied by Basu and colleagues (1989) animals that were vitamin A deficient, our animals had sufficient levels of vitamin A provided in the diet in addition to carrot enrichment. This may have influenced the lack of effect seen in our animals receiving carrot enrichment.

Diverse studies have demonstrated that RBP4 levels are elevated in Type 2 diabetes, obesity and cardiovascular diseases (Yang et al., 2005; Yao-Borengasser et al., 2007). Recently, cell culture work has found that RBP4 concentrations might induce apoptosis, which may explain findings that humans with renal dysfunction and even diabetic patients with microalbuminuria have increased RBP4 levels (Raila et al., 2007). Findings from humans have now seen that fatty infiltration by itself in the liver induces changes in RBP4 levels, which is consistent with some of the animal literature (Lanne et al., 2006), suggesting that different results for RBP4 might be explained in part by differences in the level of RBP4 and PPARγ regulation between rats and different experimental models of systemic stress (Casillas-Ramirez et al., 2011). Consequently, we speculate that increased expression of TTR band at 42kDA and of RBP4 may be an early adaptation to diabetic stress. Future study should investigate the protein levels of these retinol transporters over time, to see if disease progression is coordinated with a subsequent fall in expression, if results are animal model dependent, and if renal damage or hepatic steatosis are truly reflective of RBP4's upregulation.

4. Influence of carrot powder on serum vitamin A

4.1. Influence of carrot powder on serum vitamin A levels

Serum levels of vitamin A were measured to assess the impact of carrot diet as well as liver retinoid status. Animals fed the carrot diet had higher retinol than their healthy (13%) and diabetic (9%) counterparts, but trends did not reach significance. Our reported serum retinol concentrations were within the upper range (0.45-1.75 μmol/L) of previously reported values in the literature (Duncan et al., 1993; van Vliet et al., 1996). This is inspite of using differing extraction and chromatography conditions, as no consensus exists for a standard extraction procedure. We anticipated that our serum retinol concentrations would be higher, as previous studies showed that vitamin A or β-carotene therapy increased Vitamin A status after induction of its deficiency in healthy rodent models (Goswami et al., 2003). Instead, our study used the platform of vitamin A sufficiency before providing carrot powder enrichment (15% w/w), which constitutes a dose exceeding normal human consumption. Interestingly, one recent trial of papaya and jackfruit supplementation, rich in β-carotene, reported higher serum retinol concentrations in the supplementation groups (20% dry weight) compared to controls (Chandrika et al., 2005). Additionally, classic studies of vitamin A assessment in diabetes conducted by reported lower plasma retinol concentrations in diabetes after 4 weeks, paired with lower retinal within the retina (Tuitoek et al., 1996b; Tuitoek et al., 1996c), whereas this was not the case in our study where no differences were found. Human trials have also suggested that low retinol status in Type 1 diabetic children (Martinoli et al., 1993) may be the result of increased utilization of vitamin A as an antioxidant to eliminate free radical stress; however this theory remains unproven.

As the analyzed serum was collected under fasting conditions, this may have impacted the representation of carotenoids and retinol derivatives (such as α-retinol) which are primarily transported by chylomicrons (Dever et al., 2011; Quadro et al., 2003).

In fasting, retinol-RBP complex accounts for approximately 99% of all retinoids present in the blood (Quadro et al., 2003). Beta-carotene and α-carotene were not detected in our samples, as no post-meal chylomicrons and chylomicron remnants would be within the system. This alternate pathway of distribution explains why knockout mouse models of RBP and TTR (RBP-/-, TTR-/-) have demonstrated normal tissue concentrations of vitamin A and normal vision with vitamin A supplementation present in a normal chow diet (Episkopou et al., 1993; Quadro et al., 1999). This may further explain why we reported no statistically different concentrations of all-*trans*-retinal and all-*trans*-retinol within the retina, as chylomicron delivery of carotenoids and vitamin A derivatives may have contributed to uptake, when utilization within the eye may be altered. This may partially explain why in early stage diabetic retinopathy systemic vitamin A distribution appears to be sufficient.

Loss of body adiposity, which was evident in both diabetic groups, may also explain normal serum retinol levels. In humans, reductions in body composition, specifically fat mass associated with lifestyle changes and Roux-en-Y bypass surgery, have been related to increases in serum concentrations of lutein and β-carotene (Boesing et al., 2010; Kirby et al., 2011). This suggests that body fat, at least in humans, may act as a reservoir for carotenoids, and that weight loss can positively influence circulating carotenoid levels, and perhaps ultimately increase availability to extrahepatic tissues.

At the same time, the literature describing how the retina, or more specifically how the retinal pigment epithelium (RPE) acquires retinol is very limited. Vogel (2002) found that the RPE has limited ability to accept retinyl esters from chylomicrons by using vitamin A tracers, but never considered the impact of carotenoids β-carotene and α-carotene. Meanwhile, another research group has found that visual responsiveness was maintained in mice lacking functional RBP, but carried a transgene that expressed human

RBP (Quadro et al., 2002). High amounts of holo RPB (hRBP) were secreted into blood, and no functional defect was found upon ERG assessment, yet no hRBP was found in the RPE. Consequently, the authors were unsure how vitamin A was being taken up by the RPE, when extrahepatic hRBP efficiently delivered it to the eye to rescue vision. Along with the recent findings of α-retinol RBP independent uptake into the retina (Dever et al., 2011), it suggests that still very little is known about the mechanisms by which the retina receives sufficient substrate for the visual cycle.

4.2 Influence of carrot powder on serum vitamin A transporters

Regardless of its cause, long term repression of retinol, RBP and/or TTR can lead to complications in vision (Rosales et al., 1996). As previously mentioned, in a fasted state, RBP4 is the primary transporter of retinol to extrahepatic tissues. In our ELISA findings, serum RBP4 was found to be significantly lower in only the normal control group compared to other groups. Values fell within reported kit values for rats and mice. Resultantly it meant that both carrot-enrichment and early onset diabetic damage influenced protein levels. Carrot powder enrichment may have influenced increased levels of the protein in serum. Biobreeding diabetic prone (BBdp) rats spontaneously developing Type 1 diabetes supplemented with retinyl palmitate had a change in their plasma RBP4 concentrations, which was coupled with the elevation in hepatic RBP4 mRNA (Lu et al., 2000a). The remaining literature on serum and plasma RBP4 and TTR is very mixed. Studies of adult identical twin pairs discordant for Type 1 observed the average mean serum retinol of Type 1 diabetics to be 46ug/dl, whereby their healthy matched pairs had levels of 60.9µg/dl (Dubrey et al., 1997). Concurrently they found levels of RBP reduced to 6.2mg/dl compared with 7.6mg/dl in the healthy controls. Meanwhile other studies of Type 1 diabetic children have shown only a reduction in plasma concentrations of retinol, but not RBP4 (Baena et al., 2002; Hozumi et al., 1998). Differences in serum and liver RBP levels may be related to length and severity of Type 1

diabetes onset, however additional studies are required for confirmation as we only measured RBP at one timepoint.

The serum ELISA results do not reflect the upregulation seen in liver transporters. A 2007 study assessed the performance of assays for detecting changes in human serum RBP4 of healthy and Type 2 diabetic insulin resistance (a condition which increases RBP4 serum levels) (Graham et al., 2007). After comparing quantitative Western blotting and three commercially available multi-well immunoassays, they determined that Western blotting may be superior for discerning RBP4 levels in normal subjects from elevated levels in insulin-resistant subjects, as there was less overlap than with the immunoassays. Western blotting was deemed the current "gold standard". Immunoassays trended towards over-report of RBP4 levels among insulin-sensitive and normal populations. Authors attributed this to increased background signal of the assay and differences in reactivity between full-length RBP4 proteins, although in our assay the standards were made from mouse serum, which should more comparable to our samples than their kits using mouse urine. TTR interference may also play a role in the accuracy of our RBP4 assay as there is no denaturation step in ELISA conditions. However, we utilized ELISA in lieu of Western blots for serum as non-specific binding to both primary and secondary antibodies is a limitation of serum analysis. In our Western blotting technique a limitation is the denaturation step that separates RBP4 from TTR, making it impossible to speculate as to any level of binding in vivo required for retinol distribution and extrahepatic uptake. Fed and fasting states may also play a role on serum results, and require additional testing in order to determine differences.

Significance of Research

This is the first study to test the effects of carrot powder as a potential therapeutic agent in diabetic retinopathy. It served as a pilot study to explore the effects of

enrichment on retinal structure and function as well as vitamin A distribution in the diabetic retina, uniquely examining retinoid concentrations in conjunction with ERG outcomes. Based on the retinal immunofluorescence and immunohistochemistry, our STZ-induced animals were found to have early stage diabetic retinopathy. While carrot powder increased b-wave ON-bipolar cell activity in healthy state, it had a detrimental effect as indicated by reduced ERG response at 15% (w/w) in an STZ-induced diabetic rat model. This may suggest that the oxidative stress of a diabetic environment in combination with 15% (w/w) carrot powder supplementation triggers functional damage to the retina. Additionally, it appears that carrot powder enrichment in diabetes is paired with reduced conversion of β-carotene and α-carotene to vitamin A, with accumulation appearing in the liver tissue, which has never been assessed. However these findings are not strongly associated with the retinol levels of the retina and serum. The link between liver size, steatosis and vitamin A enzyme/receptors should be explored and confirmed in the next stage of the project as it is another novel finding from the study. Additionally, vitamin A enzyme and transport protein levels and markers of oxidative stress should be examined with a range of carotenoid enrichment doses within the retina to determine a mechanism of damage.

Strengths & limitations

This is the first known study to assess retina function and carotenoid distribution in response to whole carrot powder enrichment in healthy and diabetic animals. Our feeding paradigm was adopted to establish the effects of carrot powder conversion in the presence of vitamin A sufficiency. We established a diet capable of providing carrot in its carotenoid-rich food form in lieu of a purified form, with the intention of having a more representative model of dietary metabolism. Another major strength of the present research was the development of a new technique of carotenoid analysis using ultra

performance liquid chromatography and a C30 column. It enabled us to systematically detect and quantify retinoids and carotenoids in our samples, which was critical in the assessment of tissue concentrations. We were unable to identify retinyl esters other than retinyl palmitate, as no commercially available standards of retinyl laurate, oleate or stearate were available at the time of analysis. They were previously quantified in rat liver tissues by Schaffer and Das (2010) using a C18 column. However due to column type, and use of completely different mobile phase chromatographic conditions, comparison between our work and that of the authors was not possible in order to identify the smaller unknown peaks in our chromatograms.

Several limitations are inherent to our exploratory study design. Firstly, the use of a whole food makes it impossible for us to dismiss that one or more constituents of the product may have resulted in our findings. It is assumed that the observed effects of carrot powder were due to the carotenoid composition. Other biologically active components may have been present in the powder, but were not investigated. Additionally, although we were able to assess visual function outcomes and tissue concentrations, we did not have sufficient sample size to test specific enzymes, oxidative stress and transporters known to be involved in carotenoid and retinoid metabolism in the retina. Consequently, we are unable to draw conclusions regarding the mechanism of action involved in visual impairment in diabetes, or visual improvement in health imparted by the results. In addition, our feeding paradigm was undertaken to ensure there would be perceived effect to visual function and to establish proof of functional differences in a model of diabetic retinopathy. Consequently, the diet provided a large dose of carrot powder (15% w/w) prior to diabetes onset which would most accurately represent a population traditionally consuming large amounts of carrots over their lifetime with supplementation. Our dose is not traditionally achievable in humans by diet alone, and is a higher level dietary dose (3.65x greater vitamin A than recommended, assuming 12:1 β-carotene, and 24:1 α-

carotene conversion in rats). In turn, loss of visual function in diabetes may indicate a differing threshold of tolerance and toxicity depending on disease state. Our study revealed a detrimental effect of supplementation on retinopathy which is in support of results from previous large human interventional trials ATBC and CARET which found high doses of purified β-carotene provided daily increased incidence of lung cancer and perpetuated oxidative stress in populations who were already exposed to high levels of carcinogens and oxidative stress (Albanes et al., 1996; Omenn et al., 1994) and recent in vivo work of carotenoid oxidation products (Amengual et al., 2011).

Furthermore, as an uncontrolled model of Type 1 diabetes mellitus, insulin was not provided at any time during the study. Consequently, we cannot discount any role the lack of insulin may have independently played on energy and vitamin metabolism. Furthermore, our model used only male animals, as such data may not accurately portray findings in a female population as sex hormones estrogen and androgen have been known to impact gene expression, as well as vitamin A and β-carotene tissue variations (van Helden et al., 2011).

Lastly, duration of diabetes greater than 9 weeks would be more suitable in future studies of diabetic retinopathy, as they may yield more pronounced results. In our study only functional changes as reported by the ERG were present, with low amounts of oxidative stress and prior to pronounced signs of classical structural damage to the retina could be seen. Twelve weeks of STZ-induced diabetes (Phipps et al., 2006) may garner progressed disease pathophysiology.

Recommendations for future research

There are several options for follow-up to this study to further enhance understanding about the effects of carrot powder supplementation on diabetic metabolism, complications, and more specifically retinopathy. My first recommendation

is to assess dose response relative to carrot powder supplementation and visual function by ERG outcomes. Our feeding paradigm was adopted to ensure maximum tissue effects and to establish proof of protective effect of carotenoid conversion in the presence of vitamin A sufficiency in a diabetic model. Consequently, we provided a large dose, prior to onset that would enable us to see any protective effect, rather than interventional effects. We used a level of carrot powder supplementation previously used in study of cardiovascular complications of diabetes which is 3.65x higher than recommended intake of vitamin A for rats at a 12:1 β-carotene and 24:1 α-carotene conversion, and would qualify as an uncommon level of human intake (Nicolle et al., 2003). Although the previous study authors reported no detrimental effects, there may be differing thresholds in disease which may have contributed to the loss of ERG amplitude in the diabetic group. What we may be reporting is a toxic dose, which contributes to systemic damage in a state of diabetic oxidation and inflammation. As previously mentioned, these effects may be comparable to what was seen in human studies which used high dose synthetic β-carotene supplementation and saw an increased incidence of lung cancer and damage in environments of great oxidative stress (20mg per day vs. normal intake of 2-4mg) (Albanes et al., 1996; Kataja-Tuomola et al., 2010; Omenn et al., 1994). Consequently, an assessment of lower doses, starting at 5% (w/w) is required for future consideration, and for a better understanding carotenoid metabolism and influence on retinopathy.

Due to small sample size and time constraints, we were unable to assess oxidative stress, AGEs or IRBP transporter function within the retina; Any of which may have contributed to the loss of ERG amplitude in the diabetic group. Future research should consider correlating ERG results with these measurements, particularly as little is known about carotenoid transportation and utilization within the retina. Oxidized breakdown products of carotenoids may in fact have a role in ocular tissue dysfunction and examination is required. A number of studies in the recent past have demonstrated that

oxidized carotenoid products could cause cyto- and geno-toxicity, however they have not all been proven in animal models. In vitro, lutein-derived products-induced toxicity in RPE cells as well as post-translational protein modification (Kalariya et al., 2009; Kalariya et al., 2011). These facts are consistent with the results from previous studies which have demonstrated that A2E (a component of human retinal lipofuscin with structural analogy to carotenoids) non-fluorescing oxidized products can damage RPE cells as well as generate aldehydes, ketones, and epoxides which could be highly reactive (Dillon et al., 2004). It is conceivable that the antioxidant effect of carotenoids at high doses may be overwhelmed by oxidative stress causing oxidation products in the retina of diabetic patients with poor glycemic control. Thus understanding the role of carotenoid oxidation products and oxidative stress levels in ocular tissues must be assessed to determine the true therapeutic use of carrot powder in retinopathy.

The inter-relationship between carrot powder supplementation in diabetes and lipid metabolism should further be explored. Our study identified a relationship between increasing liver size in diabetes and carrot powder supplementation. The presence or absence of steatosis requires confirmation. Liver lipid droplet size and distribution requires histological examination. Lipid profile, especially lipoprotein levels of patients with Type 1 diabetes have been shown to be highly dependent on glycemic control. In poorly controlled Type 1 diabetes, reduced HDL is common (Ginsberg, 1996). Hepatic lipase is also suggested to be reduced by insulin deficiency. Without hepatic lipase, there is a lower clearance of postprandial remnant lipoproteins (Ruotolo et al., 1994; Tavangar et al., 1992). Knowing that the main route of transportation of carotenoids and α-retinol, derived from α-carotene, are chylomicron mediated (Dever et al., 2011), suggests the importance of investigation of lipid metabolism in our animal model. Moreover, expression and protein levels of BCDO2 should be considered in future study. This protein is known to cleave xanthophylls and carotenoids asymmetrically, and protect the

mitrochodria from carotenoid toxicity-induced dysfunctions (Amengual et al., 2011). As BCDO2 deficiency has been known to produce accumulation of triacylglycerides and liver steatosis in animals supplemented with carotenoids, its role should also be considered in our animal model.

Conclusions

Under normal conditions, carotenoid supplementation is beneficial to the retina. Under pathological conditions such as in diabetic retinopathy (which is associated with oxidative stress), carotenoid supplementation at 15% (w/w) may exert a detrimental effect. It remains to be confirmed as to whether the increase in ERG amplitudes in healthy animals suggests improved visual acuity; consequently, future studies should consider testing optokinetic reflex, which measures smooth pursuit and saccade eye movements. Conversely, ERG impairment seen in diabetic animals may be reflective of a higher availability of carotenoid for chromophore involved in phototranduction activation, inner retinal vascular angiogensis, or susceptibility to oxidation. At present, the underlying mechanisrn of impaired visual function with carrot powder enrichment in uncontrolled Type 1 diabetes is still not fully understood. Although previous studies have suggested a need for vitamin A supplementation in Type 1 diabetes patients with marginal serum retinol levels in view of its possible preventive effect on certain long-term diabetes complications (Granado et al. 1998), there is little evidence to confirm that carrot powder supplementation has any benefit. Considering the impaired hepatic conversion of β-carotene and α-carotene existing in Type 1 diabetes, and the potential for accumulation of oxidized by-products, supplementation of carotenoids by carrot powder at high concentration used in this study cannot be recommended at present time.

Summary

Although carrot has been known for better vision, no studies have been tested its effects on retinal function. This is the first study to assess the direct effects of whole carrot powder in health and in the circumstances of diabetes. It served as a pilot study to explore the effects of dietary carrot enrichment on retinal structure and function as well as vitamin A and carotenoid availability in the retina. Loss of ERG oscillatory potentials, a typical phenomenon in diabetes, with normal histology indicated that animals were at early stage diabetic retinopathy. The major finding is that animals provided carrot powder as part of a diet (15%, w/w diet) trended toward improved rod- and cone driven retinal function in healthy conditions, and yet it was detrimental in STZ-induced Type 1 diabetes. Additionally, it appears that carrot powder supplementation in diabetes is paired with β-carotene and α-carotene accumulation and lower levels of retinol and retinyl palmitate in the liver tissue, suggesting conversion impairment which may affect vitamin A metabolism downstream in the retina. However these findings were not strongly associated with the retinol levels of retina and serum. Considering the impact on ERG response, impaired hepatic conversion of β-carotene and α-carotene existing in Type 1 diabetes, and the potential for accumulation of carotenoid by-products, carrot powder supplementation at 15% (w/w diet) cannot presently be recommended as a preventative therapy for diabetic retinopathy in Type 1 diabetes.

Literature Cited

Abahusain MA, Wright J, Dickerson JW, de Vol EB. 1999. Retinol, alpha-tocopherol and carotenoids in diabetes. Eur J Clin Nutr 53:630-635.

Akbaraly TN, Fontbonne A, Favier A, Berr C. 2008. Plasma carotenoids and onset of dysglycemia in an elderly population: Results of the epidemiology of vascular ageing study. Diabetes Care 31:1355-1359.

Albanes D, Heinonen OP, Taylor PR *et al.* 1996. Alpha-tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: Effects of base-line characteristics and study compliance. J Natl Cancer Inst 88:1560-1570.

Alper G, Irer S, Duman E, Caglayan O, Yilmaz C. 2005. Effect of I-deprenyl and gliclazide on oxidant stress/antioxidant status and dna damage in a diabetic rat model. Endocr Res 31:199-212.

Amengual J, Lobo GP, Golczak M, Li HN, Klimova T, Hoppel CL, Wyss A, Palczewski K, von Lintig J. 2011. A mitochondrial enzyme degrades carotenoids and protects against oxidative stress. FASEB J 25:948-959.

Araszkiewicz A, Naskret D, Niedzwiecki P, Samborski P, Wierusz-Wysocka B, Zozulinska-Ziolkiewicz D. 2011. Increased accumulation of skin advanced glycation end products is associated with microvascular complications in type 1 diabetes. Diabetes Technol Ther 13:837-842.

Arnal E, Miranda M, Barcia J, Bosch-Morell F, Romero FJ. 2010. Lutein and docosahexaenoic acid prevent cortex lipid peroxidation in streptozotocin-induced diabetic rat cerebral cortex. Neuroscience 166:271-278.

Arnal E, Miranda M, Johnsen-Soriano S, Alvarez-Nolting R, Diaz-Llopis M, Araiz J, Cervera E, Bosch-Morell F, Romero FJ. 2009. Beneficial effect of docosahexanoic acid and lutein on retinal structural, metabolic, and functional abnormalities in diabetic rats. Curr Eye Res 34:928-938.

Aylward GW, Billson FA. 1989. The scotopic threshold response in diabetic retinopathy-a preliminary report. Aust N Z J Ophthalmol 17:369-372.

Baena RM, Campoy C, Bayes R, Blanca E, Fernandez JM, Molina-Font JA. 2002. Vitamin A, retinol binding protein and lipids in type 1 diabetes mellitus. Eur J Clin Nutr 56:44-50.

Barker FM. 2010. Dietary supplementation: Effects on visual performance and occurrence of AMD and cataracts. Curr Med Res Opin 26:2011-2023.

Basu TK, Basualdo C. 1997. Vitamin A homeostasis and diabetes mellitus. Nutrition 13:804-806.

Basu TK, Tze WJ, Leichter J. 1989. Serum vitamin A and retinol-binding protein in patients with insulin-dependent diabetes mellitus. Am J Clin Nutr 50:329-331.

Batten ML, Imanishi Y, Maeda T, Tu DC, Moise AR. 2004. Lecithin-retinol acyltransferase is essential for accumulation of all-trans-retinyl esters in the eye and in the liver. J Biol Chem 279:10422.

Baynes JW. 2001. The role of AGEs in aging: Causation or correlation. Exp Gerontol 36:1527-1537.

Bendich A, Olson JA. 1989. Biological actions of carotenoids. FASEB J 3:1927-1932.

Berdanier CD, Everts HB, Hermoyian C, Mathews CE. 2001. Role of vitamin A in mitochondrial gene expression. Diabetes Res Clin Pract 54 Suppl 2:S11-27.

Berkowitz BA, Bissig D, Patel P, Bhatia A, Roberts R. 2012. Acute systemic 11-cis-retinal intervention improves abnormal outer retinal ion channel closure in diabetic mice. Mol Vis 18:372-376.

Bernstein PS, Khachik F, Carvalho LS, Muir GJ, Zhao DY, Katz NB. 2001. Identification and quantitation of carotenoids and their metabolites in the tissues of the human eye. Exp Eye Res 72:215-223.

Bhatti RA, Yu S, Boulanger A, Fariss RN, Guo Y, Bernstein SL, Gentleman S, Redmond TM. 2003. Expression of beta-carotene 15,15' monooxygenase in retina and RPE-choroid. Invest Ophthalmol Vis Sci 44:44-49.

Blaner WS, O'Byrne SM, Wongsiriroj N, Kluwe J, D'Ambrosio DM, Jiang H, Schwabe RF, Hillman EM, Piantedosi R, Libien J. 2009. Hepatic stellate cell lipid droplets: A specialized lipid droplet for retinoid storage. Biochim Biophys Acta 1791:467-473.

Blomhoff R, Green MH, Berg T, Norum KR. 1990. Transport and storage of vitamin A. Science 250:399.

Boesing F, Moreira EA, Wilhelm-Filho D, Vigil SV, Parizottto EB, Inacio DB, Portari GV, Trindade EB, Jordao-Junior AA, Frode TS. 2010. Roux-en-Y bypass gastroplasty: Markers of oxidative stress 6 months after surgery. Obes Surg 20:1236-1244.

Brazionis L, Rowley K, Itsiopoulos C, O'Dea K. 2009. Plasma carotenoids and diabetic retinopathy. Br J Nutr 101:270-277.

Brownlee M. 2005. The pathobiology of diabetic complications: A unifying mechanism. Diabetes 54:1615-1625.

Brucklacher RM, Patel KM, VanGuilder HD *et al.* 2008. Whole genome assessment of the retinal response to diabetes reveals a progressive neurovascular inflammatory response. BMC Med Genomics 1:26.

Bui BV, Armitage JA, Tolcos M, Cooper ME, Vingrys AJ. 2003. ACE inhibition salvages the visual loss caused by diabetes. Diabetologia 46:401-408.

Bulum T, Kolaric B, Duvnjak L, Duvnjak M. 2011. Nonalcoholic fatty liver disease markers are associated with insulin resistance in type 1 diabetes. Dig Dis Sci 56:3655-3663.

Burri BJ, Clifford AJ. 2004. Carotenoid and retinoid metabolism: Insights from isotope studies. Arch Biochem Biophys 430:110-119.

Cai SF, Kirby RJ, Howles PN, Hui DY. 2001. Differentiation-dependent expression and localization of the class B type I scavenger receptor in intestine. J Lipid Res 42:902.

Calloway DH, Murphy SP, Beaton GH, Lein D. 1993. Estimated vitamin intakes of toddlers: Predicted prevalence of inadequacy in village populations in Egypt, Kenya, and Mexico. Am J Clin Nutr 58:376-384.

Cano J, Machado A, Reinoso-Suarez F. 1986. Morphological changes in the retina of ageing rats. Arch Gerontol Geriatr 5:41-50.

Cardinault N, Tyssandier V, Grolier P, Winklhofer-Roob BM, Ribalta J, Bouteloup-Demange C, Rock E, Borel P. 2003. Comparison of the postprandial chylomicron carotenoid responses in young and older subjects. Eur J Nutr 42:315-323.

Carpentier S, Knaus M, Suh M. 2009. Associations between lutein, zeaxanthin, and age-related macular degeneration: An overview. Crit Rev Food Sci Nutr 49:313-326.

Casillas-Ramirez A, Alfany-Fernandez I, Massip-Salcedo M, Juan ME, Planas JM, Serafin A, Pallas M, Rimola A, Rodes J, Peralta C. 2011. Retinol-binding protein 4 and peroxisome proliferator-activated receptor-gamma in steatotic liver transplantation. J Pharmacol Exp Ther 338:143-153.

Castenmiller JJ, West CE. 1998. Bioavailibility and bioconverstion of carotenoids. Annu Rev Nutr 18:19.

Chandrika U, Jansz E, Warnasuriya N. 2005. Analysis of carotenoids in ripe jackfruit (*artocarpus heterophyllus*) kernel and study of their bioconversion in rats. J. Sci. Food Agric 85:186-190.

Charng J, Nguyen CT, Bui BV, Vingrys AJ. 2011. Age-related retinal function changes in albino and pigmented rats. Invest Ophthalmol Vis Sci

Chaturvedi N, Fuller JH, Taskinen MR, EURODIAB PCS Group. 2001. Differing associations of lipid and lipoprotein disturbances with the macrovascular and microvascular complications of type 1 diabetes. Diabetes Care 24:2071-2077.

Chen M, Thomson AB, Tsin AT, Basu TK. 2003. The hepatic retinyl ester hydrolase activity is depressed at the onset of diabetes in BB rats. Br J Nutr 89:231-238.

Chen YH, Oace SM, Wolf G. 1999. Studies on the effect of dose size on the absorption of beta-carotene by the rat in vivo. Int J Vitam Nutr Res 69:8-15.

Chertow BS, Blaner WS, Baranetsky NG, Sivitz WI, Cordle MB, Thompson D, Meda P. 1987. Effects of vitamin A deficiency and repletion on rat insulin secretion in vivo and in vitro from isolated islets. J Clin Invest 79:163-169.

Chichili GR, Nohr D, Schaffer M, von Lintig J, Biesalski HK. 2005. Beta-carotene conversion into vitamin A in human retinal pigemnt epithelial cells. Invest Ophthalmol Vis Sci 46:3562.

Christensen EI, Moskaug JO, Vorum H, Jacobsen C, Gundersen TE, Nykjaer A, Blomhoff R, Willnow TE, Moestrup SK. 1999. Evidence for an essential role of megalin in transepithelial transport of retinol. J Am Soc Nephrol 10:685-695.

Congdon NG, Friedman DS, Lietman T. 2003. Important causes of visual impairment in the world today. JAMA 290:2057-2060.

Czernichow S, Couthouis A, Bertrais S, Vergnaud AC, Dauchet L, Galan P, Hercberg S. 2006. Antioxidant supplementation does not affect fasting plasma glucose in the supplementation with antioxidant vitamins and minerals (SU.VI.MAX) study in France: Association with dietary intake and plasma concentrations. Am J Clin Nutr 84:395-399.

Dal-Pizzol F, Klamt F, Benfato MS, Bernard EA, Moreira JC. 2001. Retinol supplementation induces oxidative stress and modulates antioxidant enzyme activities in rat sertoli cells. Free Radic Res 34:395-404.

Dene BA, Maritim AC, Sanders RA, Watkins JB,3rd. 2005. Effects of antioxidant treatment on normal and diabetic rat retinal enzyme activities. J Ocul Pharmacol Ther 21:28-35.

Desobry SA, Netto FM, Labuza TP. 1998. Preservation of beta-carotene from carrots. Crit Rev Food Sci Nutr 38:381-396.

Dever JT, Surles RL, Davis CR, Tanumihardjo SA. 2011. Alpha-retinol is distributed through serum retinol-binding protein-independent mechanisms in the lactating sow-nursing piglet dyad. J Nutr 141:42-47.

Dillon J, Wang Z, Avalle LB, Gaillard ER. 2004. The photochemical oxidation of A2E results in the formation of a 5,8,5',8'-bis-furanoid oxide. Exp Eye Res 79:537-542.

Ditzel J. 1976. Oxygen transport impairment in diabetes. Diabetes 25:832-838.

Dixon JL, Goodman DS. 1987. Studies on the metabolism of retinol-binding protein by primary hepatocytes from retinol-deficient rats. J Cell Physiol 130:14-20.

Dubrey SW, Beetham R, Miles J, Noble MI, Rowe R, Leslie RD. 1997. Increased urinary albumin and retinol-binding protein in type I diabetes. A study of identical twins. Diabetes Care 20:84-89.

Dueker SR, Lin Y, Buchholz BA, Schneider PD, Lame MW, Segall HJ, Vogel JS, Clifford AJ. 2000. Long-term kinetic study of beta-carotene, using accelerator mass spectrometry in an adult volunteer. J Lipid Res 41:1790-1800.

Duncan TE, Green JB, Green MH. 1993. Liver vitamin A levels in rats are predicted by a modified isotope dilution technique. J Nutr 123:933-939.

During A, Doraiswamy S, Harrison EH. 2008. Xanthophylls are preferentially taken up compared with beta-carotene by retinal cells via a SRBI-dependent mechanism. J Lipid Res 49:1715.

During A, Harrison EH. 2007. Mechanisms of provitamin A (carotenoid) and vitamin A (retinol) transport into and out of intestinal caco-2 cells. J Lipid Res 48:2283.

Egeland GM, Berti P, Soueida R, Arbour LT, Receveur O, Kuhnlein HV. 2004. Age differences in vitamin A intake among Canadian inuit. Can J Public Health 95:465-469.

Episkopou V, Maeda S, Nishiguchi S, Shimada K, Gaitanaris GA, Gottesman ME, Robertson EJ. 1993. Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. Proc Natl Acad Sci U S A 90:2375-2379.

Escaron AL, Tanumihardjo SA. 2006. Absorption and transit of lutein and beta-carotene supplements in the mongolian gerbil (Meriones unguiculatus). Int J Vitam Nutr Res 76:315-323.

Espe K, Galler A, Raila J, Kiess W, Schweigert FJ. 2007. High-normal C-reactive protein levels do not affect the vitamin A transport complex in serum of children and adolescents with type 1 diabetes. Pediatr Res 62:741-745.

Filipek S, Stenkamp RE, Teller DC, Palczewski K. 2003. G protein-coupled receptor rhodopsin: A prospectus. Annu Rev Physiol 65:851-879.

Ford ES, Will JC, Bowman BA, Narayan KM. 1999. Diabetes mellitus and serum carotenoids: Findings from the third national health and nutrition examination study. Am J Epidemiol 149:168.

Fraser PD, Pinto ME, Holloway DE, Bramley PM. 2000. Technical advance: Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. Plant J 24:551-558.

Gardiner TA, Anderson HR, Stitt AW. 2003. Inhibition of advanced glycation end-products protects against retinal capillary basement membrane expansion during long-term diabetes. J Pathol 201:328-333.

Gelain DP, de Bittencourt Pasquali MA, Caregnato FF, Moreira JC. 2011. Vitamin A (retinol) up-regulates the receptor for advanced glycation endproducts (RAGE) through p38 and akt oxidant-dependent activation. Toxicology 289:38-44.

Gerhardinger C, Costa MB, Coulombe MC, Toth I, Hoehn T, Grosu P. 2005. Expression of acute-phase response proteins in retinal muller cells in diabetes. Invest Ophthalmol Vis Sci 46:349-357.

Gillman MW, Cupples LA, Gagnon D, Posner BM, Ellison RC, Castelli WP, Wolf PA. 1995. Protective effect of fruits and vegetables on development of stroke in men. JAMA 273:1113-1117.

Ginsberg HN. 1996. Diabetic dyslipidemia: Basic mechanisms underlying the common hypertriglyceridemia and low HDL cholesterol levels. Diabetes 45 Suppl 3:S27-30.

Goh SY, Cooper ME. 2008. Clinical review: The role of advanced glycation end products in progression and complications of diabetes. J Clin Endocrinol Metab 93:1143-1152.

Goodman DS. 1984. Overview of current knowledge of metabolism of vitamin A and carotenoids. J Natl Cancer Inst 73:1375-1379.

Goswami BC, Ivanoff KD, Barua AB. 2003. Absorption and conversion of 11,12-(3)H-beta-carotene to vitamin A in sprague-dawley rats of different vitamin A status. J Nutr 133:148-153.

Graham TE, Wason CJ, Bluher M, Kahn BB. 2007. Shortcomings in methodology complicate measurements of serum retinol binding protein (RBP4) in insulin-resistant human subjects. Diabetologia 50:814-823.

Greenberg ER, Sporn MB. 1996. Antioxidant vitamins, cancer, and cardiovascular disease. N Engl J Med 334:1189-1190.

Guerin CJ, Anderson DH, Fisher SK. 1990. Changes in intermediate filament immunolabeling occur in response to retinal detachment and reattachment in primates. Invest Ophthalmol Vis Sci 31:1474-1482.

Gutteridge JM, Halliwell B. 1982. The role of the superoxide and hydroxyl radicals in the degradation of DNA and deoxyribose induced by a copper-phenanthroline complex. Biochem Pharmacol 31:2801-2805.

Hammes HP. 2005. Pericytes and the pathogenesis of diabetic retinopathy. Horm Metab Res 37 Suppl 1:39-43.

Hammes HP, Alt A, Niwa T, Clausen JT, Bretzel RG, Brownlee M, Schleicher ED. 1999. Differential accumulation of advanced glycation end products in the course of diabetic retinopathy. Diabetologia 42:728-736.

Hammes HP, Bartmann A, Engel L, Wulfroth P. 1997. Antioxidant treatment of experimental diabetic retinopathy in rats with nicanartine. Diabetologia 40:629-634.

Han Y, Adams AJ, Bearse MA, Schneck ME. 2004. Multifocal electroretinogram and short-wavelength automated perimetry measures in diabetic eyes with little or no retinopathy. Arch Ophthalmol 122:1809-1815.

He CJ, Koschinsky T, Buenting C, Vlassara H. 2001. Presence of diabetic complications in type 1 diabetic patients correlates with low expression of mononuclear cell AGE-receptor-1 and elevated serum AGE. Mol Med 7:159-168.

Health Canada. 2010. Reference values for vitamins. 2010. http://www.hc-sc.gc.ca/fn-an/nutrition/reference/table/ref_vitam_tbl-eng.php

Health Canada. 2009a. Canadian community health survey, cycle 2.2 nutrition: Vitamin A.

Health Canada. 2009b. Canadian nutrient file: Raw, carrot. 2010. http://webprod3.hc-sc.gc.ca/cnf-fce/index-eng.jsp

Heinonen MI. 1990. Carotenoid and provitamin A activity of carrot cultivars. J Agric Food Chem 609-612.

Hennekens CH, Buring JE, Manson JE *et al.* 1996. Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease. N Engl J Med 334:1145-1149.

Hilmantel G, Applegate RA, van Heuven WA, Stowers SP, Bradley A, Lee BL. 1999. Entoptic foveal avascular zone measurement and diabetic retinopathy. Optom Vis Sci 76:826-831.

Hoppe PP, Kramer K, van den Berg H, Steenge G, van Vliet T. 2003. Synthetic and tomato-based lycopene have identical bioavailability in humans. Eur J Nutr 42:272-278.

Hozumi M, Murata T, Morinobu T, Manago M, Kuno T, Tokuda M, Konishi K, Mingci Z, Tamai H. 1998. Plasma beta-carotene, retinol, and alpha-tocopherol levels in relation to glycemic control of children with insulin-dependent diabetes mellitus. J Nutr Sci Vitaminol 44:1-9.

Imanishi Y, Batten ML, Piston DW, Baehr W, Palczewski K. 2004. Noninvasive two-photon imaging reveals retinyl ester storage structures in the eye. J Cell Biol 164:373-383.

International Diabetes Federation. 2010. Diabetes in the young: A global perspective.

Isken A, Golczak M, Oberhauser V, Hunzelmann S, Driever W, Imanishi Y, Palczewski K, von Lintig J. 2008. RBP4 disrupts vitamin A uptake homeostasis in a STRA6-deficient animal model for matthew-wood syndrome. Cell Metab 7:258-268.

Jawa A, Kcomt J, Fonseca VA. 2004. Diabetic nephropathy and retinopathy. Med Clin North Am 88:1001-36.

Jha P, Flather M, Lonn E, Farkouh M, Yusuf S. 1995. The antioxidant vitamins and cardiovascular disease. A critical review of epidemiologic and clinical trial data. Ann Intern Med 123:860-872.

Joussen AM, Smyth N, Niessen C. 2007. Pathophysiology of diabetic macular edema. Dev Ophthalmol 39:1-12.

Kalariya NM, Ramana KV, Srivastava SK, van Kuijk FJ. 2011. Post-translational protein modification by carotenoid cleavage products. Biofactors 37:104-116.

Kalariya NM, Ramana KV, Srivastava SK, van Kuijk FJ. 2009. Genotoxic effects of carotenoid breakdown products in human retinal pigment epithelial cells. Curr Eye Res 34:737-747.

Kalea AZ, Schmidt AM, Hudson BI. 2009. RAGE: A novel biological and genetic marker for vascular disease. Clin Sci (Lond) 116:621-637.

Kanwar M, Chan PS, Kern TS, Kowluru RA. 2007. Oxidative damage in the retinal mitochondria of diabetic mice: Possible protection by superoxide dismutase. Invest Ophthalmol Vis Sci 48:3805-3811.

Karachalias N, Babaei-Jadidi R, Ahmed N, Thornalley PJ. 2003. Accumulation of fructosyl-lysine and advanced glycation end products in the kidney, retina and peripheral nerve of streptozotocin-induced diabetic rats. Biochem Soc Trans 31:1423-1425.

Kataja-Tuomola MK, Kontto JP, Mannisto S, Albanes D, Virtamo JR. 2010. Effect of alpha-tocopherol and beta-carotene supplementation on macrovascular complications and total mortality from diabetes: Results of the ATBC study. Ann Med 42:178-186.

Khachik F, Bernstein PS, Garland DL. 1997. Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. Invest Ophthalmol Vis Sci 38:1802-1811.

Kiefer C, Hessel S, Lampert JM, Vogt K, Lederer MO. 2001. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. J Biol Chem 276:14110.

Kirby ML, Beatty S, Stack J, Harrison M, Greene I, McBrinn S, Carroll P, Nolan JM. 2011. Changes in macular pigment optical density and serum concentrations of lutein and zeaxanthin in response to weight loss. Br J Nutr 105:1036-1046.

Knekt P, Reunanen A, Jarvinen R, Seppanen R, Heliovaara M, Aromaa A. 1994. Antioxidant vitamin intake and coronary mortality in a longitudinal population study. Am J Epidemiol 139:1180-1189.

Kowluru A, Kowluru RA, Yamazaki A. 1992. Functional alterations of G-proteins in diabetic rat retina: A possible explanation for the early visual abnormalities in diabetes mellitus. Diabetologia 35:624-631.

Kowluru RA, Kanwar M, Chan PS, Zhang JP. 2008a&b. Inhibition of retinopathy and retinal metabolic abnormalities in diabetic rats with AREDS-based micronutrients. Arch Ophthalmol 126:1266-1272.

Kowluru RA, Koppolu P, Chakrabarti S, Chen S. 2003. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. Free Radic Res 37:1169-1180.

Kowluru RA, Menon B, Gierhart DL. 2008. Beneficial effect of zeaxanthin on retinal metabolic abnormalities in diabetic rats. Invest Ophthalmol Vis Sci 49:1645-1651.

Kowluru RA, Odenbach S. 2004. Effect of long-term administration of alpha-lipoic acid on retinal capillary cell death and the development of retinopathy in diabetic rats. Diabetes 53:3233-3238.

Krill D, O'Leary L, Koehler AN, Kramer MK, Warty V, Wagner MA, Dorman JS. 1997. Association of retinol binding protein in multiple-case families with insulin-dependent diabetes. Hum Biol 69:89-96.

Krinsky NI. 1989. Antioxidant functions of carotenoids. Free Radic Biol Med 7:617-635.

Kurlandsky SB, Gamble MV, Ramakrishnan R, Blaner WS. 1995. Plasma delivery of retinoic acid to tissues in the rat. J Biol Chem 270:17850-17857.

Lai YL, Jacoby RO, Jonas AM. 1978. Age-related and light-associated retinal changes in fischer rats. Invest Ophthalmol Vis Sci 17:634-638.

Lanne B, Dahllof B, Lindahl C *et al.* 2006. PPARalpha and PPARgamma regulation of liver and adipose proteins in obese and dyslipidemic rodents. J Proteome Res 5:1850-1859.

Leeds JS, Forman EM, Morley S, Scott AR, Tesfaye S, Sanders DS. 2009. Abnormal liver function tests in patients with type 1 diabetes mellitus: Prevalence, clinical correlations and underlying pathologies. Diabet Med 26:1235-1241.

Lemke SL, Dueker SR, Follett JR, Lin Y, Carkeet C, Buchholz BA, Vogel JS, Clifford AJ. 2003. Absorption and retinol equivalence of beta-carotene in humans is influenced by dietary vitamin A intake. J Lipid Res 44:1591-1600.

Li G, Tang J, Du Y, Lee CA, Kern TS. 2011. Beneficial effects of a novel RAGE inhibitor on early diabetic retinopathy and tactile allodynia. Mol Vis 17:3156-3165.

Li J, Schmidt AM. 1997. Characterization and functional analysis of the promoter of RAGE, the receptor for advanced glycation end products. J Biol Chem 272:16498-16506.

Lindqvist A, He YG, Andersson S. 2005. Cell type-specific expression of beta-carotene 15,15'-monooxygenase. J Histochem Cytochem 53:1403.

Liu C, Wang XD, Mucci L, Gaziano JM, Zhang SM. 2009. Modulation of lung molecular biomarkers by beta-carotene in the physicians' health study. Cancer 115:1049-1058.

Lobo GP, Hessel S, Eichinger A, Noy N, Moise AR, Wyss A, Palczewski K, von Lintig J. 2010. ISX is a retinoic acid-sensitive gatekeeper that controls intestinal beta,beta-carotene absorption and vitamin A production. FASEB J 24:1656-1666.

Low JW, Arimond M, Osman N, Cunguara B, Zano F, Tschirley D. 2007. A food-based approach introducing orange-fleshed sweet potatoes increased vitamin A intake and serum retinol concentrations in young children in rural mozambique. J Nutr 137:1320-1327.

Lu J, Dixon WT, Tsin AT, Basu TK. 2000a. The metabolic availability of vitamin A is decreased at the onset of diabetes in BB rats. J Nutr 130:1958-1962.

Lu J, Field CJ, Basu TK. 2000b. The immune responses to diabetes in BB rats supplemented with vitamin A. J Nutr Biochem 11:515-520.

MacDonald PN, Ong DE. 1988. Evidence for a lecithin-retinol acyltransferase activity in the rat small intestine. J Biol Chem 263:12478.

Maeda A, Maeda T, Golczak M, Chou S, Desai A, Hoppel CL, Matsuyama S, Palczewski K. 2009. Involvement of all-trans-retinal in acute light-induced retinopathy of mice. J Biol Chem 284:15173-15183.

Maeda A, Maeda T, Sun W, Zhang H, Baehr W, Palczewski K. 2007. Redundant and unique roles of retinol dehydrogenases in the mouse retina. Proc Natl Acad Sci U S A 104:19565-19570.

Maiani G, Caston MJ, Catasta G *et al.* 2009. Carotenoids: Actual knowledge on food sources, intakes, stability and bioavailability and their protective role in humans. Mol Nutr Food Res 53 Suppl 2:S194-218.

Mares JA, LaRowe TL, Snodderly DM, Moeller SM, Gruber MJ, Klein ML, Wooten BR, Johnson EJ, Chappell RJ, CAREDS Macular Pigment Study Group and Investigators. 2006. Predictors of optical density of lutein and zeaxanthin in retinas of older women in the carotenoids in age-related eye disease study, an ancillary study of the women's health initiative. Am J Clin Nutr 84:1107-1122.

Martinoli L, Di Felice M, Seghieri G, Ciuti M, De Giorgio LA, Fazzini A, Gori R, Anichini R, Franconi F. 1993. Plasma retinol and alpha-tocopherol concentrations in insulin-dependent diabetes mellitus: Their relationship to microvascular complications. Int J Vitam Nutr Res 63:87-92.

Mata NL, Radu RA, Clemmons RC, Travis GH. 2002. Isomerization and oxidation of vitamin a in cone-dominant retinas: A novel pathway for visual-pigment regeneration in daylight. Neuron 36:69-80.

Mata NL, Ruiz A, Radu RA, Bui TV, Travis GH. 2005. Chicken retinas contain a retinoid isomerase activity that catalyzes the direct conversion of all-trans-retinol to 11-cis-retinol. Biochemistry 44:11715-11721.

Mathews-Roth MM. 1988. Lack of genotoxicity with beta-carotene. Toxicol Lett 41:185-191.

Matthews KA, Rhoten WB, Driscoll HK, Chertow BS. 2004. Vitamin A deficiency impairs fetal islet development and causes subsequent glucose intolerance in adult rats. J Nutr 134:1958-1963.

Miranda M, Muriach M, Johnsen S, Bosch-Morell F, Araiz J, Roma J, Romero FJ. 2004. Oxidative stress in a model for experimental diabetic retinopathy: Treatment with antioxidants. Arch Soc Esp Oftalmol 79:289-294.

Mizutani M, Gerhardinger C, Lorenzi M. 1998. Müller cell changes in human diabetic retinopathy. Diabetes 47:445-449.

Moiseyev G, Takahashi Y, Chen Y, Gentleman S, Redmond TM. 2006. RPE65 is an iron(II)-dependent isomerohydrolase in the retinoid visual cycle. J Biol Chem 281:2835.

Murano I, Morroni M, Zingaretti MC, Oliver P, Sanchez J, Fuster A, Pico C, Palou A, Cinti S. 2005. Morphology of ferret subcutaneous adipose tissue after 6-month daily supplementation with oral beta-carotene. Biochim Biophys Acta 1740:305-312.

Murata F, Horie I, Ando T *et al.* 2012. A case of glycogenic hepatopathy developed in a patient with new-onset fulminant type 1 diabetes: The role of image modalities in diagnosing hepatic glycogen deposition including gradient-dual-echo MRI. Endocr J

Murata T, Nagai R, Ishibashi T, Inomuta H, Ikeda K, Horiuchi S. 1997. The relationship between accumulation of advanced glycation end products and expression of vascular endothelial growth factor in human diabetic retinas. Diabetologia 40:764-769.

Mustafi D, Engel AH, Palczewski K. 2009. Structure of cone photoreceptors. Prog Retin Eye Res 28:289-302.

Nicolle C, Cardinault N, Aprikian O *et al.* 2003. Effect of carrot intake on cholesterol metabolism and on antioxidant status in cholesterol-fed rat. Eur J Nutr 42:254-261.

Nollevaux MC, Guiot Y, Horsmans Y, Leclercq I, Rahier J, Geubel AP, Sempoux C. 2006. Hypervitaminosis A-induced liver fibrosis: Stellate cell activation and daily dose consumption. Liver Int 26:182-186.

Olson JA. 1984. Serum levels of vitamin A and carotenoids as reflectors of nutritional status. J Natl Cancer Inst 73:1439-1444.

Olson JA, Shike M, Shils ME, editors. 1999. Modern nutrition in health and disease: Carotenoids. Baltimore: Williams & Wilkins. 525-41 p.

Omenn GS, Goodman G, Grizzle J, Thornquist M, Rosenstock L, Barnhart S, Anderson G, Balmes J, Cherniack M, Cone J. 1991. CARET, the beta-carotene and retinol efficacy trial to prevent lung cancer in asbestos-exposed workers and in smokers. Anticancer Drugs 2:79-86.

Omenn GS, Goodman G, Thornquist M, Grizzle J, Rosenstock L, Barnhart S, Balmes J, Cherniack MG, Cullen MR, Glass A. 1994. The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: Smokers and asbestos-exposed workers. Cancer Res 54:2038s-2043s.

Ottlecz A, Garcia CA, Eichberg J, Fox DA. 1993. Alterations in retinal na+, K(+)-ATPase in diabetes: Streptozotocin-induced and zucker diabetic fatty rats. Curr Eye Res 12:1111-1121.

Owsley C, McGwin G, Jackson GR, Heimburger DC, Piyathilake CJ, Klein R, White MF, Kallies K. 2006. Effect of short-term, high-dose retinol on dark adaptation in aging and early age-related maculopathy. Invest Ophthalmol Vis Sci 47:1310-1318.

Pasquali MA, Gelain DP, Zanotto-Filho A, de Souza LF, de Oliveira RB, Klamt F, Moreira JC. 2008. Retinol and retinoic acid modulate catalase activity in sertoli cells by distinct and gene expression-independent mechanisms. Toxicol in Vitro 22:1177-1183.

Pelikanova T. 2007. Pathogenesis of diabetic retinopathy. Vnitr Lek 53:498-505.

Penniston KL, Tanumihardjo SA. 2006. The acute and chronic toxic effects of vitamin A. Am J Clin Nutr 83:191-201.

Peterson PA, Rask L. 1971. Studies on the fluorescence of the human vitamin A-transporting plasma protein complex and its individual components. J Biol Chem 246:7544-7550.

Phipps JA, Fletcher EL, Vingrys AJ. 2004. Paired-flash identification of rod and cone dysfunction in the diabetic rat. Invest Ophthalmol Vis Sci 45:4592-4600.

Phipps JA, Yee P, Fletcher EL, Vingrys AJ. 2006. Rod photoreceptor dysfunction in diabetes: Activation, deactivation, and dark adaptation. Invest Ophthalmol Vis Sci 47:3187-3194.

Qazi Y, Maddula S, Ambati BK. 2009. Mediators of ocular angiogenesis. J Genet 88:495-515.

Quadro L, Blaner WS, Hamberger L, Van Gelder RN, Vogel S, Piantedosi R, Gouras P, Colantuoni V, Gottesman ME. 2002. Muscle expression of human retinol-binding protein (RBP). suppression of the visual defect of RBP knockout mice. J Biol Chem 277:30191-30197.

Quadro L, Blaner WS, Salchow DJ, Vogel S, Piantedosi R, Gouras P, Freeman S, Cosma MP, Colantuoni V, Gottesman ME. 1999. Impaired retinal function and vitamin A availability in mice lacking retinol-binding protein. EMBO J 18:4633-4644.

Quadro L, Hamberger L, Colantuoni V, Gottesman ME, Blaner WS. 2003. Understanding the physiological role of retinol-binding protein in vitamin A metabolism using transgenic and knockout mouse models. Mol Aspects Med 24:421-430.

Raila J, Henze A, Spranger J, Mohlig M, Pfeiffer AF, Schweigert FJ. 2007. Microalbuminuria is a major determinant of elevated plasma retinol-binding protein 4 in type 2 diabetic patients. Kidney Int 72:505-511.

Redmond TM, Gentleman S, Duncan T, Yu S, Wiggert B. 2001. Identification, expression, and substrate specificity of a mammalian beta-carotene 15,15'-dioxygenase. J Biol Chem 276:6560.

Redmond TM, Poliakov E, Yu S, Tsai JY, Lu Z, Gentleman S. 2005. Mutation of key residues of RPE65 abolishes its enzymatic role as isomerohydrolase in the visual cycle. Proc Natl Acad Sci 102:13658-13663.

Ribaya-Mercado JD, Holmgren SC, Fox JG, Russell RM. 1989. Dietary beta-carotene absorption and metabolism in ferrets and rats. J Nutr 119:665-668.

Robbins J. 2002. Transthyretin from discovery to now. Clin Chem Lab Med 40:1183-1190.

Rock CL, Swendseid ME, Jacob RA, McKee RW. 1992. Plasma carotenoid levels in human subjects fed a low carotenoid diet. J Nutr 122:96-100.

Rosales FJ, Ritter SJ, Zolfaghari R, Smith JE, Ross AC. 1996. Effects of acute inflammation on plasma retinol, retinol-binding protein, and its mRNA in the liver and kidneys of vitamin A-sufficient rats. J Lipid Res 37:962-971.

Rosenfalck AM, Almdal T, Hilsted J, Madsbad S. 2002. Body composition in adults with type 1 diabetes at onset and during the first year of insulin therapy. Diabet Med 19:417-423.

Ruotolo G, Parlavecchia M, Taskinen MR, Galimberti G, Zoppo A, Le NA, Ragogna F, Micossi P, Pozza G. 1994. Normalization of lipoprotein composition by intraperitoneal insulin in IDDM. role of increased hepatic lipase activity. Diabetes Care 17:6-12.

Rytter E, Vessby B, Asgard R, Ersson C, Moussavian S, Sjodin A, Abramsson-Zetterberg L, Moller L, Basu S. 2010. Supplementation with a combination of antioxidants does not affect glycaemic control, oxidative stress or inflammation in type 2 diabetes subjects. Free Radic Res 44:1445-1453.

Samardzija M, von Lintig J, Tanimoto N, Oberhauser V, Thiersch M. 2008. R91W mutation in RPE65 leads to milder early-onset retinal dystrophy due to the generation of low levels of 11-cis-retinal. Hum Mol Genet 17:281.

Sasaki M, Ozawa Y, Kurihara T, Kubota S, Yuki K, Noda K, Kobayashi S, Ishida S, Tsubota K. 2010. Neurodegenerative influence of oxidative stress in the retina of a murine model of diabetes. Diabetologia 53:971-979.

Sauberlich HE, Hodges RE, Wallace DL, Kolder H, Canham JE, Hood J, Raica N,Jr, LOWRY LK. 1974. Vitamin A metabolism and requirements in the human studied with the use of labeled retinol. Vitam Horm 32:251-275.

Schaffer MW, Roy SS, Mukherjee S, Nohr D, Wolter M, Biesalski HK, Ong DE, Das SK. 2010. Qualitative and quantitative analysis of retinol, retinyl esters, tocopherols and selected carotenoids out of various internal organs form different species by HPLC. Anal Methods 2:1320-1332.

Schmidt AM, Hori O, Chen JX, Li JF, Crandall J, Zhang J, Cao R, Yan SD, Brett J, Stern D. 1995. Advanced glycation endproducts interacting with their endothelial receptor induce expression of vascular cell adhesion molecule-1 (VCAM-1) in cultured human endothelial cells and in mice. A potential mechanism for the accelerated vasculopathy of diabetes. J Clin Invest 96:1395-1403.

Seino Y, Miki T, Kiyonari H, Abe T, Fujimoto W. 2008. ISX participates in the maintenance of vitamin A metabolism by regulation of beta-carotene 15,15'-monooxygenase expression. J Biol Chem 283:4905.

Shidfar F, Aghasi M, Vafa M, Heydari I, Hosseini S, Shidfar S. 2010. Effects of combination of zinc and vitamin A supplementation on serum fasting blood sugar, insulin, apoprotein B and apoprotein A-I in patients with type I diabetes. Int J Food Sci Nutr 61:182-191.

Shirao Y, Kawasaki K. 1998. Electrical responses from diabetic retina. Prog Retin Eye Res 17:59-76.

Simo R, Villarroel M, Corraliza L, Hernandez C, Garcia-Ramirez M. 2010. The retinal pigment epithelium: Something more than a constituent of the blood-retinal barrier--implications for the pathogenesis of diabetic retinopathy. J Biomed Biotechnol 2010:190724.

Sinha A, Formica C, Tsalamandris C, Panagiotopoulos S, Hendrich E, DeLuise M, Seeman E, Jerums G. 1996. Effects of insulin on body composition in patients with insulin-dependent and non-insulin-dependent diabetes. Diabet Med 13:40-46.

Stitt AW. 2010. AGEs and diabetic retinopathy. Invest Ophthalmol Vis Sci 51:4867-4874.

Stitt AW, Bhaduri T, McMullen CB, Gardiner TA, Archer DB. 2000. Advanced glycation end products induce blood-retinal barrier dysfunction in normoglycemic rats. Mol Cell Biol Res Commun 3:380-388.

Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H. 1997. Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. Am J Pathol 150:523-531.

Suh M, Sauve Y, Merrells KJ, Kang JX, Ma DW. 2009. Supranormal electroretinogram in fat-1 mice with retinas enriched in docosahexaenoic acid and n-3 very long chain fatty acids (C24-C36). Invest Ophthalmol Vis Sci 50:4394-4401.

Szel A, Rohlich P, Caffe AR, van Veen T. 1996. Distribution of cone photoreceptors in the mammalian retina. Microsc Res Tech 35:445-462.

Targher G, Bertolini L, Chonchol M, Rodella S, Zoppini G, Lippi G, Zenari L, Bonora E. 2010. Non-alcoholic fatty liver disease is independently associated with an increased prevalence of chronic kidney disease and retinopathy in type 1 diabetic patients. Diabetologia 53:1341-1348.

Tavangar K, Murata Y, Pedersen ME, Goers JF, Hoffman AR, Kraemer FB. 1992. Regulation of lipoprotein lipase in the diabetic rat. J Clin Invest 90:1672-1678.

The World Health Organization (WHO). 2009. Global prevalence of vitamin A deficiency in populations at risk 1995–2005: WHO global database on vitamin A deficiency.

Thornalley PJ. 1998. Cell activation by glycated proteins. AGE receptors, receptor recognition factors and functional classification of AGEs. Cell Mol Biol (Noisy-Le-Grand) 44:1013-1023.

Thornalley PJ, Langborg A, Minhas HS. 1999. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem J 344 Pt 1:109-116.

Tong L, Vernon SA. 2000. Passing the DVLA field regulations following bilateral macular photocoagulation in diabetics. Eye (Lond) 14:35-38.

Tuitoek PJ, Lakey JR, Rajotte RV, Basu TK. 1996a. Strain variation in vitamin A (retinol) status of streptozotocin-induced diabetic rats. Int J Vitam Nutr Res 66:101-105.

Tuitoek PJ, Ritter SJ, Smith JE, Basu TK. 1996b. Streptozotocin-induced diabetes lowers retinol-binding protein and transthyretin concentrations in rats. Br J Nutr 76:891-897.

Tuitoek PJ, Ziari S, Tsin AT, Rajotte RV, Suh M, Basu TK. 1996c. Streptozotocin-induced diabetes in rats is associated with impaired metabolic availability of vitamin A (retinol). Br J Nutr 75:615-622.

van Helden YG, Godschalk RW, von Lintig J, Lietz G, Landrier JF, Luisa Bonet M, van Schooten FJ, Keijer J. 2011. Gene expression response of mouse lung, liver and white adipose tissue to beta-carotene supplementation, knockout of Bcmo1 and sex. Mol Nutr Food Res 55:1466-1474.

van Vliet T, van Vlissingen MF, van Schaik F, van den Berg H. 1996. Beta-carotene absorption and cleavage in rats is affected by the vitamin A concentration of the diet. J Nutr 126:499-508.

Vivat-Hannah V, Zusi FC. 2005. Retinoids as therapeutic agents: Today and tomorrow. Mini Rev Med Chem 5:755-760.

Vogel S, Piantedosi R, O'Byrne SM, Kako Y, Quadro L, Gottesman ME, Goldberg IJ, Blaner WS. 2002. Retinol-binding protein-deficient mice: Biochemical basis for impaired vision. Biochemistry 41:15360-15368.

von Lintig J, Vogt K. 2000. Filling the gap in vitamin A research. molecular identification of an enzyme cleaving beta-carotene to retinal. J Biol Chem 275:11915.

von Lintig J, Kiser PD, Golczak M, Palczewski K. 2010. The biochemical and structural basis for trans-to-cis isomerization of retinoids in the chemistry of vision. Trends Biochem Sci 35:400-410.

Voshol PJ, Schwarz M, Rigotti A, Krieger M, Groen AK, Kuipers F. 2001. Down-regulation of intestinal scavenger receptor class B, type I expression in rodents under conditions of deficient bile delivery to the intestine. Biochem J 356:317.

Wake K. 1980. Perisinusoidal stellate cells (fat-storing cells, interstitial cells, lipocytes), their related structure in and around the liver sinusoids, and vitamin A-storing cells in extrahepatic organs. Int Rev Cytol 66:303-353.

Wang XD, Krinsky NI. 1998. The bioconversion of beta-carotene into retinoids. Subcell Biochem 30:159-180.

Wang Y, Roger Illingworth D, Connor SL, Barton Duell P, Connor WE. 2010. Competitive inhibition of carotenoid transport and tissue concentrations by high dose supplements of lutein, zeaxanthin and beta-carotene. Eur J Nutr 49:327-336.

West KP,Jr, Christian P, Katz J, Labrique A, Klemm R, Sommer A. 2010. Effect of vitamin A supplementation on maternal survival. Lancet 376:873-4; author reply 874.

Wiggert B, Chader GJ. 1985. Monkey interphotoreceptor retinoid-binding protein (IRBP): Isolation, characterization and synthesis. Prog Clin Biol Res 190:89-110.

World Health Organization. 1976. Vitamin A deficiency and xerophthalmia.

Yan SD, Stern D, Schmidt AM. 1997. What's the RAGE? the receptor for advanced glycation end products (RAGE) and the dark side of glucose. Eur J Clin Invest 27:179-181.

Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB. 2005. Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. Nature 436:356-362.

Yao-Borengasser A, Varma V, Bodles AM *et al.* 2007. Retinol binding protein 4 expression in humans: Relationship to insulin resistance, inflammation, and response to pioglitazone. J Clin Endocrinol Metab 92:2590-2597.

Yee P, Weymouth AE, Fletcher EL, Vingrys AJ. 2010. A role for omega-3 polyunsaturated fatty acid supplements in diabetic neuropathy. Invest Ophthalmol Vis Sci 51:1755-1764.

Zhang X, Lai Y, McCance DR, Uchida K, McDonald DM, Gardiner TA, Stitt AW, Curtis TM. 2008. Evaluation of N (epsilon)-(3-formyl-3,4-dehydropiperidino)lysine as a novel biomarker for the severity of diabetic retinopathy. Diabetologia 51:1723-1730.

Appendix A

1. Experimental design:

1.1. Animal Housing & Feed Measurement

Housing also contained shavings, cardboard tube, and Nylabone chew toy. Principles of laboratory animal care were followed. Feed intake was measured from study start as a weekly two-day food record captured from Monday AM to Wednesday AM.

2. Serum glucose

Reaction:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic Acid} + H_20_2$$

Chemicals, materials and preparation of standard stock solutions:

Glucose colour reagent, a solution reconstituted with 100 mL distilled H_2O also contained a buffer (pH 7.25 at 25ºC), 0.25 mmol/L 4-aminoantipyrine, 20 mmol/L p-hydroxybenzoate, >40,000 U/L glucose oxidase (microbial), >200 U/L peroxidase, and preservatives.

As a calibrant, glucose standard (5 mmol/L) was serially diluted to produce 4 additional standards at concentrations of 2.5, 1.25, 0.625, 0.3125 mmol/L.

Sample preparation & procedure:

Aliquoted termination serum was thawed on ice, vortexed, and diluted with distilled H_2O (5x for non-diabetic, 20x for early diabetic animals). Quality control (DC-TROL Level 2, Genzyme Diagnostics, Charlottetown, PEI, Cat.#SM-052) was reconstituted with 5 mL of distilled H_2O (1:9 dilution) in the same manner as the samples. Blank, standards, quality control, and samples were vortexed and 5 µL of each were

plated in triplicate on a 96-well plate (Costar, Corning, New York, Cat.#3370). A randomly selected serum sample was run on each plate as a reference of inter-plate consistency. Two hundred µl of the glucose colour reagent was added to each well. The plate was covered with a lid and incubated for 10 minutes at room temperature. Following incubation, absorbance was read at 505 nm using a FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany) and Omega Control Software (Version 1.0, BMG Labtech, Offenburg, Germany).

For all assays, results were multiplied by the dilution factor where applicable to obtain the concentration of the parameter of interest. The following conditions were met by the assay; correlation coefficients from standard curves of each plate were equal or greater than 0.95, with a coefficient of variation less than 10% for all standards, controls, and samples. All samples fell within the range of the standard curve.

3. Liquid chromatography of retinoids and carotenoids

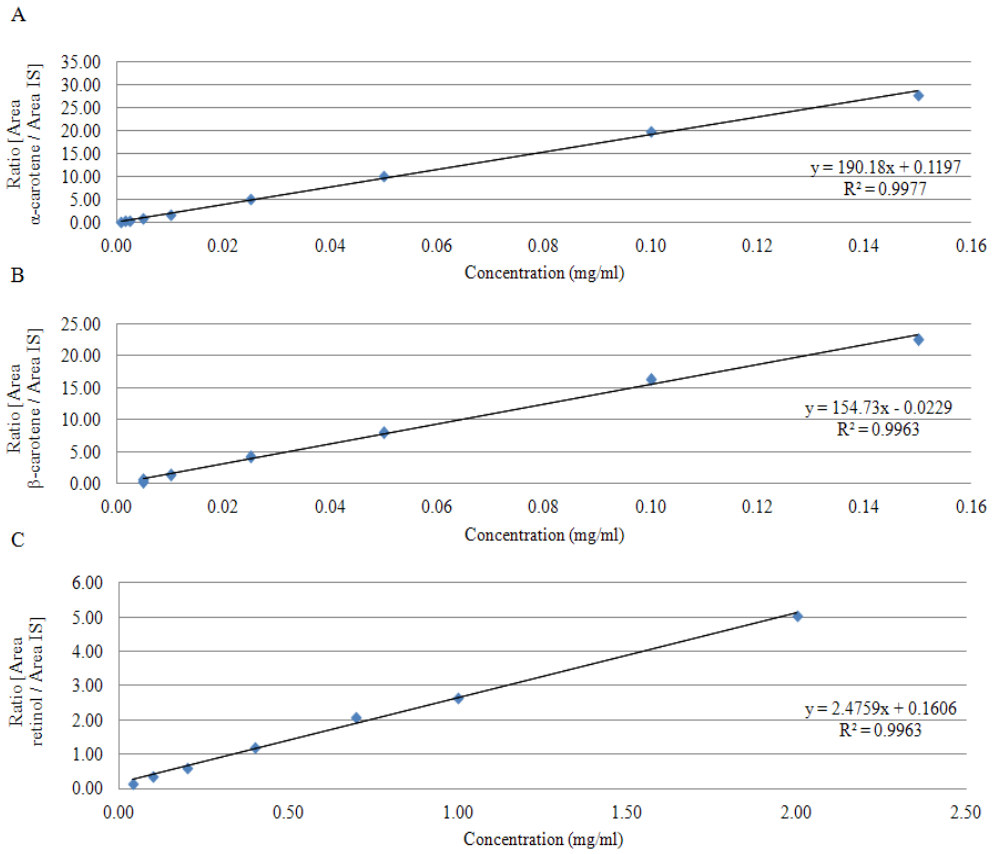

Figure A-1: Sample calibration curves for α-carotene, β-carotene and retinol for liver tissue based on calibration standards. Concentrations expressed as the ratio between the compound and the internal standard retinyl acetate.

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

Tissue	Compound	Equations	R Value
Liver	α-carotene	y = 208.08x - 0.1256	R^2 = 0.9977
Liver	β-carotene	y = 154.73x - 0.0229	R^2 = 0.9963
Liver	retinol	y = 2.4759x + 0.1606	R^2 = 0.9963
Liver	r. palmitate	y = 2.1882x + 0.0194	R^2 = 0.9896
Serum	retinol	y = 4.6774x + 0.0011	R^2 = 0.9920
Retina	retinal	y = 0.1218x + 0.0076	R^2 = 0.9928
Retina	retinol	y = 2.121x + 0.0078	R^2 = 0.9996

4. Enzyme-linked immunosorbent assay of RBP4 in serum

The AdipoGen (Cat.#AG-45A-0012EK-KI01, San Diego, CA) assay kit utilized a pre-coated polyclonal antibody microplate with specific detection for RBP4. Once washed, horseradish peroxidase conjugated anti-rabbit IgG was added as the detector. Following final washing and incubation, the concentration of RBP4 was quantified with the addition of 3,3',5,5'-tetramethylbenzidine (TMB) based on the proportional intensity of color developed in the substrate solution measured at its absorbance of 450±10 nm following acidification.

Chemicals, materials and preparation of standard stock solutions:

The kit supplied mouse serum RBP4 stock (24ng/ml) which was reconstituted with 1ml of deionized water, aliquoted and stored at -20C until use. Seven tubes of calibrant were prepared using 2-fold serial dilutions (12, 6, 3, 1.5, 0.75, 0.375, 0.188 and 0 (blank) ng/mL). Mouse RBP4 QC sample was reconstituted with 1 ml of deionized water and was allowed to sit for 15 minutes prior to use.

Reagents:

- Assay Diluent A (1x) was reconstituted by diluting 100mL of Assay Diluent A Concentrate (10x) with 200mL of distilled water.

- Detection Reagent (HRP Conjugated anti-rabbit IgG) (1x) was reconstituted by adding 120µl of Detection Reagent (100x) to 12ml of Assay Diluent A (1x).

- Wash solution (1x) was prepared by adding 450mL of distilled water to 50mL of Wash solution Concentrate (10x), for a 1x solution.

- Substrate Solution I and II were mixed together within 15 minutes of use and protected from the light in order to prevent degradation.

- TMB substrate – amount needed.

Sample preparation & procedure:

Aliquoted termination serum from each animal of interest was thawed on ice, vortexed, and diluted with Diluent 1x reconstituted from the kit to 15,000x for all samples. Required concentrations were achieved through a series of serial dilutions. Blank, standards, quality control, and samples were vortexed and 100µL of each were plated in triplicate and all run on the same 96-well plate. Following plating, it was sealed and incubated for 1 hour at 37°C. After incubation, solution from each well was aspirated using a multichannel vacuum, and washed with 300µL of 1x wash solution. The plate was left to stand for 1 minute. Washing procedure was repeated 3 times. Upon the last wash, complete removal of liquid was achieved with adsorbent paper. 100µL of detection antibody was added to each dry well. A new plate sealer was used to cover the plate. The plate was incubated for 1 hour at 37°C.

Detection antibody from each well was aspirated, and washing procedure was

repeated 3 times. 100µL of the diluted Detector was then added to each dry well and the plate was incubated for 1 hour at 37°C.

Diluted Detector from each well was aspirated, and washing procedure was repeated 5 times. 100ul of mixed Substrate Solution I and II was added and color reaction was allowed to develop at room temperature in the dark for 20 minutes. 100µl of Stop Solution was then added to each well. Liquid was mixed by tapping the side of the plate to ensure a uniform yellow color. Absorbance of each well read at 450 nm using the FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany) and Omega Control Software (Version 1.0, BMG Labtech, Offenburg, Germany).

6. Western immunoblot analysis of RBP and TTR in liver

6.1. Protein Extraction

Liver (40mg) samples were covered with liquid nitrogen and ground with a pestle to a fine powder. Samples were sonicated in a sample buffer containing 0.2 M Tris-HCl (pH 6.8), 3% SDS, and 30% glycerol (Sonic dismembrator, Model 100, Thermo Fisher Scientific Inc., Ottawa, ON) and centrifuged for 20 min at 13000 rpm (18000 x g) at 4ºC. Lysate was separated into 3 layers: pellet, supernatant, and scum. Protein-containing supernatant was collected and stored at -80ºC until analysis. As an intensity control, one aliquot from one sample of each of the four groups was pooled together for later use.

6.2. Protein assay

A Pierce bicinchoninic acid (BCA) kit was used to determine protein concentration (Thermo Scientific, Rockford, IL, Cat.# 23227). The assay employed BCA for colorimetric detection and quantification of total protein from liver sample supernatant. A blue colored end product was produced from the reaction between the sample protein and the alkaline medium containing cuprous cations and BCA during a 30 minute incubation at 37ºC. Colorimetric quantification of protein concentration was

achieved by measuring colour intensity at 550 nm on a FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany) with Omega Control Software (Version 1.0, BMG Labtech, Offenburg, Germany).

Chemicals, materials and preparation of standard stock solutions:

- BCA™ Protein Assay Reagent A & B containing sodium carbonate, sodium bicarbonate, BCA™ detection reagent, and sodium tartrate in 0.1N sodium hydroxide

- Albumin Standard (Thermo Scientific, Rockford, IL, Prod. # 23227), containing 2.0 mg/mL bovine serum albumin in a 0.9% aqueous NaCl solution containing sodium azide.

Sample preparation & procedure:

All samples were thawed on ice and diluted with distilled H_2O (5× dilution). Standard were prepared according to kit directions using the 2.0mg/mL albumin stock and distilled H_2O (2, 1.5, 1, 0.75, 0.5, 0 mg/mL). Ten μL of protein standards and samples were plated in triplicate and 200 μL of the working reagent (50 parts reagent A, 1 part reagent B) was added to each well of a 96-well plate (Costar, Corning, New York, Cat.#3370). The plate was then wrapped in Parafilm and incubated at 37ºC for 30 minutes. Following incubation, plates were read at 550 nm on a FLUOstar Omega microplate reader (BMG Labtech, Offenburg, Germany) with Omega Control Software (Version 1.0, BMG Labtech, Offenburg, Germany).

6.3. Gel electrophoresis

Polyacrylamide stacking and separating gels were cast from the polymerization of acrylamide; N,N-methylenebisacrylamide was used to separate proteins based on their molecular weights. Heated protein samples containing SDS buffer were loaded into the

gel. Electric current was used to pull the negatively charged proteins through the gel matrix towards the positive electrode causing smaller molecular weight proteins to travel more quickly. 10ug of liver protein was loaded into a 15% separating gel was used to more readily separate smaller molecular weight proteins, including the TTR monomer (16kDa) and RBP4 (21kDa).

Chemicals, materials and preparation of solutions:

- 15% separating gel: 7.5 mL 20% acrylamide, 2.25 mL 1.5 M Tris-HCl pH 8.8, 0.15 mL distilled H_2O, 100 μL 10% ammonium persulfate, 100 μL 10% SDS, 8 μL N,N,N',N'-Tetramethylethylenediamine (TEMED) (MP Biomedicals LLC, Solon OH, Cat. # 805615)

- *5% Stacking gel:* 1 mL 20% acrylamide, 1 mL 0.5 M Tris-HCl pH 6.8, 1 mL distilled H_2O, 40 μL 10% ammonium persulfate, 40 μL 10% SDS, 10 μL TEMED

- t-amyl-alcohol

- 1:1 mixture of 10% (v/v) bromophenol blue (BPB) and 2-mercaptoethanol (β-ME)

- 5× running buffer (288 g glycine, 60.6 g tris-base, 200 mL 10% SDS, distilled H_2O to yield a final volume of 4L)

- pre-stained molecular weight marker (BenchMark #10748-010, Invitrogen)

Sample preparation & procedure:

For gel preparation, two glass plates were cleaned with methanol and distilled H_2O and separated with 1.0 mm spacers. The plates were held together with sandwich clamps and placed in a casting stand. The separating gel mixture was pipetted between the glass plates and t-amyl-alcohol poured on top to level and eliminates bubbles in the

gel during polymerization for 1 hour. T-amyl-alcohol was then rinsed off with distilled H_2O. Stacking gel was pipetted between the glass plates and a 1.0mm 15-well comb was inserted on an angle. Stacking gel was allowed to polymerize for 15 minutes. During the stacking gel polymerization, liver and retina protein samples were prepared by placing 10 µg of sample in a microcentrifuge tube based on concentrations determined in the BCA protein assay. Volume of protein sample was determined as: 10 ug of protein (to load into gel)/ protein concentration (from protein assay) in µg/µl = volume of protein sample needed (µl).

Only small volumes of protein sample were required, consequently 10 µl of 3× sample buffer was added to aid in sample loading. A 1:1 mixture of 10% BPB:β-ME (v/v) was added as 10% per each sample based on the largest sample volume to be loaded. For example, if the largest sample to be loaded was 14 µl, 1.4 µl of the 10% BPB/β-ME was added to all samples for reduction of disulphide bridges and visualization. Samples were placed in a hot water bath to denature the proteins.

The center of the electrophoresis tank was filled with working strength running buffer. For each gel, 10µl of pre-stained protein ladder (Invitrogen BenchMark #10748-010, Life Technologies, Burlington, ON) was loaded into the first well, with the intensity control sample in the last well to adjust for intensity differences from gel to gel. Following sample loading, remaining running buffer was poured into the tank and the electrophoresis apparatus containing 2 gels per tank was connected to the power supply set to 20 mA and allowed to run for approximately 1.1 hours until the dye front reached the bottom of the gel.

6.4. Transfer of proteins (Western blotting)

Proteins were transferred in an electrical field for 1 hour from the gel onto a membrane that blots the proteins from the gel under wet transfer conditions in order to achieve visualization after a series of blocking, protein-specific antibody applications and chemiluminescence.

Chemicals, materials and preparation of solutions:

- 5× transfer buffer (75.7 g Tris-base, 242.5 g glycine, distilled H_2O to yield a final volume of 4 L)

- 5× Tris-buffered saline in Tween-20 (TBST) (400 mL 1 M tris-HCl pH 7.4, 600 mL 5 M NaCl, , 10 mL Tween-20, distilled H_2O to yield a final volume of 4 L). Filter sterilized.

- methanol

Sample procedure:

After completing the electrophoresis, gels were transferred to labeled PVDF membranes which were rehydrated in methanol and equilibrated in the working strength transfer buffer (200 mL methanol, 200 mL 5× transfer buffer, distilled H_2O to yield a final volume of 1 L) for 5 minutes. Gels were transferred onto blotting paper, covered with the PVDF membrane and covered with another blotting paper, while removing all bubbles. Two fiber boards were sandwiched around the membrane and papers before being secured into a gel transfer cassette. Clamped cassettes were placed two per transfer apparatus with a stir bar, ice pack, and transfer buffer at a constant of 100 V for 1 hour.

6.5. Identification labeling of proteins

In order to achieve protein-specific banding with proportional intensity of light to concentration, a series of primary and secondary antibodies were applied. The secondary antibody containing conjugated to horseradish peroxidase (HRP) was used to bind the primary antibody. The addition of ECL kit reagent, catalyzes a non-radioactive Lumigen PS-3 reaction emitting light which is directly proportional to the amount of antigen conjugated to Horseradish Peroxidase labeled antibody when captured using FluorChemQ with AlphaViewQ image acquisition and analysis software (Alpha Innotech, Santa Clara, CA). Each membrane was exposed and captured at various time points using movie mode scanning technology to determine optimal intensity. A listing of all antibodies, concentrations, incubation and development times is available in Table A-2.

Chemicals, materials and preparation of solutions:

- 3% BSA in TBST
- 1× TBST
- 1% BSA in TBST
- Primary antibody: See Table A-2 for the comprehensive listing
- Loading control antibody Non-phosphorylated mitogen-activated protein kinase MAPK (42kDa/ 44kDa) (Cell Signaling, Danvers, MA, 2146S)
- Secondary antibody goat anti-rabbit conjugated with horseradish peroxidase (Biorad, Mississauga, ON, Cat.#170-6515)
- ECL Plus Kit™; Solution A: ECL Plus substrate solution containing tris buffer, Solution B: Stock Acridan solution in dioxane and ethanol (AmershamTM ECL

Plus Western Blotting Detection System, GE Healthcare, Buckinghamshire, UK, Cat.#RPN2132, Cat.#RPN2133)

Sample procedure:

The PVDF membrane was placed in a blotting box and blocked with 15 mL of 3% BSA in TBST on an orbital shaker for 1 hour. Following incubation, the 3% BSA was discarded and primary antibody was added based on optimal tested dilution of 1:10000 (i.e 1 µl of antibody in 10 mL 3% BSA in TBST). The membrane was placed in a blot box sealed with Parafilm on an orbital shaker overnight at 4 ºC.

Membranes were subsequently washed 4 times for 5 minutes each in 1× TBST. Secondary antibody of goat anti-rabbit was then applied (1:10000 dilution; 1 µL antibody in 10 mL 1% BSA in TBST) for 1 hour on an orbital shaker. Again, washing procedure was repeated. Following wash, membranes were dipped in Amersham ECL kit detection reagent and slowly mixed manually in an orbital motion for 10 seconds (1 membrane used 2ml of reagent A, with 50µl of reagent B). Using FluorChemQ with AlphaViewQ each membrane was exposed and captured at various time points using movie mode scanning technology to determine optimal intensity. Optimal dilutions and exposures are shown in Table A-2. Images were converted to tiff files, and the bands were analyzed using the software.

Table A-2: Western blot antibody and exposure information.

Primary	Dilution	Secondary	Molecular Wt. (kDa)	Exposure Time (min)	Catalogue #	Company
RBP4	1:10000	Rabbit	21	7.5	71661B	Novus Biologicals
TTR	1:10000	Rabbit	16, 27,42,83	7.5	50256	Novus Biologicals
MAPK 42/44	1:1000	Rabbit	42 & 44	2.5	2146S	Cell Signaling

RBP4, Retinol binding protein 4; TTR,Transthyretin

6.6. Ponceau Stain

Rapid, reversible staining with aide of Ponceau stain (1g Ponceau S, 50ml acetic acid, made up to 1L with distilled H_2O) was used to locate and briefly quantify bands obtained on the PVDF membrane. Reddish pink bands were used to roughly quantify protein loading without deleterious effects on future protein-specific banding of membranes by antibodies. Membranes were incubated in Ponceau for 1 minute, and then rinsed with distilled water to remove excess background stain before using FluorChemQ with AlphaViewQ software. Stain was completely removed by rehydrating the membranes in methanol and subsequent washes with 1xTBST. PVDF membranes were then placed in 1× TBST (1 part 5× TBST, 4 parts distilled H2O) at 4ºC for storage prior to blocking.

6.7. Stripping membranes

Membranes were stripped in order for measurement of loading control by new application of primary, secondary antibody and chemiluminescence.

Chemicals, materials and preparation of solutions:

- Stripping buffer, Restore™ Thermo Scientific stripping buffer (Thermo Scientific PI21059 #21059)

- 1× TBST

Sample procedure:

Stripping solution (5ml) was added to membranes in a plastic sealable container for 10 minutes on an orbital shaker at room temperature. The stripping solution was then discarded and procedure was repeated one more time. After the second wash, stripping solution was discarded and membranes were placed in a clean container, and rinsed with 1× TBST in three 10 minute intervals, and stored in 1× TBST at 4°C.

Appendix B

Table B-1. Weekly body weights

Week	N	NC	D	DC
1	71.19 ± 5.58	74.86 ± 6.01	106.18 ± 7.05	130.79 ± 3.71
2	129.79 ± 4.36	132.11 ± 8.24	167.11 ± 9.54	164.70 ± 6.41
3	201.14 ± 7.95	200.73 ± 18.44	230.13 ± 12.29	229.54 ± 8.87
4	259.73 ± 10.81	247.90 ± 23.47	292.65 ± 16.00	290.40 ± 22.93
5	323.14 ± 11.71	315.29 ± 31.52	286.39 ± 16.97	308.06 ± 16.36
6	370.51 ± 10.51	374.93 ± 30.73	305.69 ± 22.05	325.20 ± 16.04
7	417.00 ± 14.41	419.93 ± 33.28	306.79 ± 26.93	335.35 ± 16.21
8	441.41 ± 10.20	462.19 ± 32.56	320.54 ± 28.37	344.44 ± 20.75
9	480.23 ± 13.36	486.64 ± 37.62	328.76 ± 23.12	354.41 ± 26.02
10	499.51 ± 16.40	529.59 ± 46.29	340.45 ± 31.48	355.99 ± 29.65
11	525.20 ± 16.43	553.29 ± 41.06	338.79 ± 29.53	355.60 ± 29.27
12	540.00 ± 18.27	582.09 ± 47.33	340.71 ± 33.22	354.85 ± 35.70

All data expressed as ± SD (n=7-8 rats/group; N, NC=7, D=8, DC=8) in grams. Week 12 weights measured prior to termination and fasting**.**

Table B-2. Weekly feed intake

Week	N	NC	D	DC
1	98.54 ± 0.25	87.17 ± 0.62	119.91 ± 0.66	130.99 ± 1.21
2	181.11 ± 4.47	178.46 ± 2.61	174.13 ± 2.96	127.12 ± 1.97
3	190.87 ± 0.72	144.57 ± 5.70	188.27 ± 4.95	149.38 ± 1.58
4	NA[1]	NA[1]	191.36 ± 1.11	166.99 ± 1.38
5	176.09 ± 4.51	177.00 ± 6.40	220.41 ± 0.76	210.92 ± 3.45
6	209.05 ± 4.90	205.10 ± 4.28	255.50 ± 5.89	203.63 ± 4.53
7	245.31 ± 5.47	213.85 ± 3.96	265.69 ± 5.48	205.63 ± 3.36
8	248.77 ± 2.75	235.84 ± 10.07	248.29 ± 6.43	219.54 ± 3.38
9	256.12 ± 5.73	218.69 ± 2.40	256.24 ± 6.81	214.11 ± 6.73
10	255.46 ± 2.93	214.69 ± 4.09	NA[1]	NA[1]

Data expressed as mean ± SD (n=7-8 rats/group) in grams. [1]Data not available due to animal fasting. NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

Table B-3. Carrot powder on metabolic parameters and endpoint blood glucose in health and Type 1 diabetes

	N	NC	D	DC	Diet (P<)	Diabetes (P<)	D*D (P<)
BG (mM)	6.51 ± 1.11^{c}	7.52 ± 1.16^{c}	35.07 ± 2.65^{a}	29.87 ± 5.17^{b}	NS	<0.0001	0.041
Water (mL)	26.5 ± 5.3^{b}	36.9 ± 8.3^{b}	178 ± 29^{a}	156 ± 45^{a}	NS	<0.0001	NS
Food (g)	24.1 ± 5.6^{b}	23.7 ± 3.0^{b}	32.2 ± 6.3^{a}	28.9 ± 5.6^{a}	NS	0.0004	NS
Urine (mL)	12.2 ± 4.4^{c}	19.7 ± 6.5^{c}	213.1 ± 58.9^{a}	143 ± 55.7^{b}	0.03	<0.0001	0.01
Feces (g)	3.6 ± 0.8^{b}	4.3 ± 1.0^{b}	9.4 ± 2.2^{a}	12 ± 4.1^{a}	NS	<0.0001	NS

All data expressed as ± SD (n=7-8 rats/group). All data expressed as ± SD (n=7-8 rats/group). Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test.

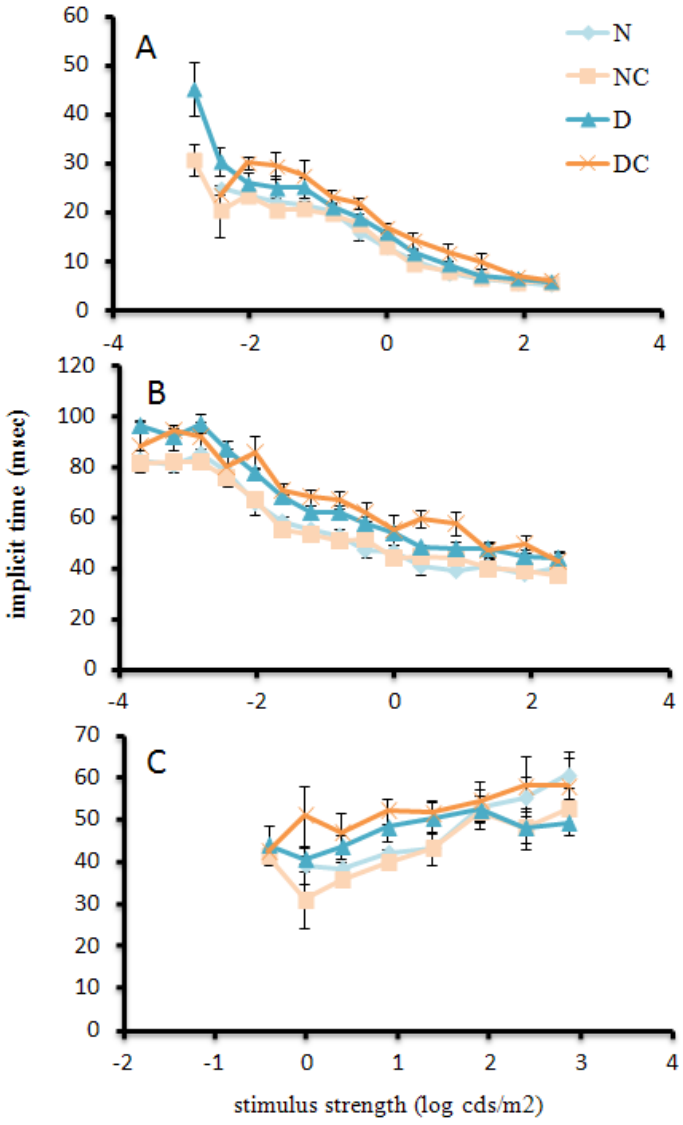

Figure B-1: Carrot powder on light and dark adapted implicit times in health and Type 1 diabetes. Data expressed as mean ± SD (n=6-8 rats/group). Significant effects of diet and disease were identified by two-way analysis of variance with Duncan's multiple range test. Scotopic response: (A) dark-adapted intensity response of a-wave implicit time; (B) dark-adapted intensity response b-wave implicit time. Photopic response: (C) light-adapted intensity response b-wave implicit time. Significant diet effects: (NS). Significant disease effects: photopic b-wave implicit time ($P<0.0004$). N, healthy without carrot enrichment; NC, healthy with carrot enrichment; D, diabetic without carrot enrichment; DC, diabetic with carrot enrichment.

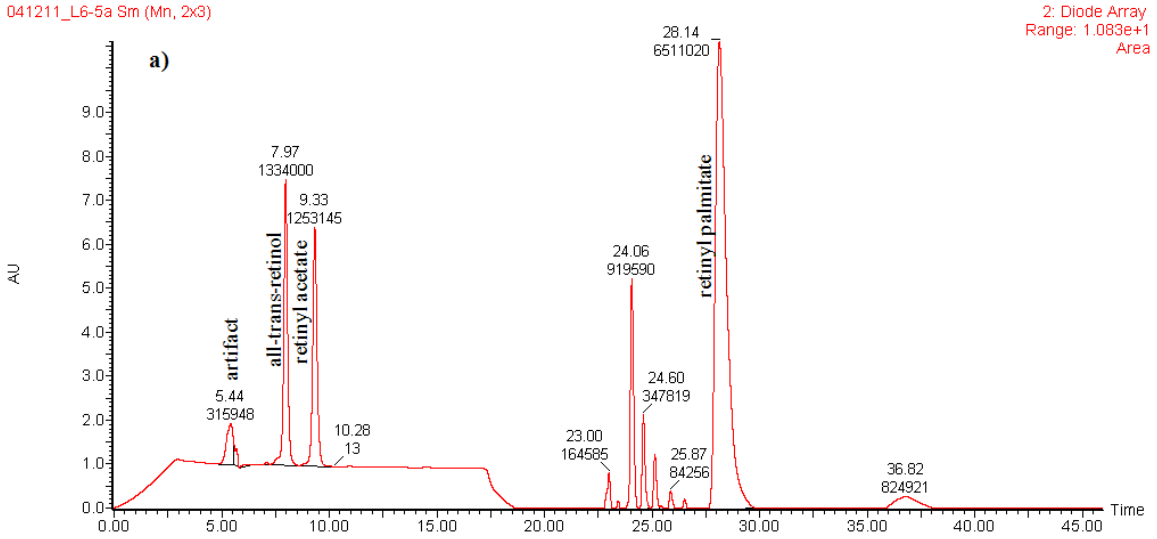

Figure B-2: Sample UPLC-PDA chromatogram for rat liver retinoids. Sample of N group containing all-*trans*-retinol, retinyl palmitate and internal standard retinyl acetate measured at the range of 320-385 nm, 10µl injection.

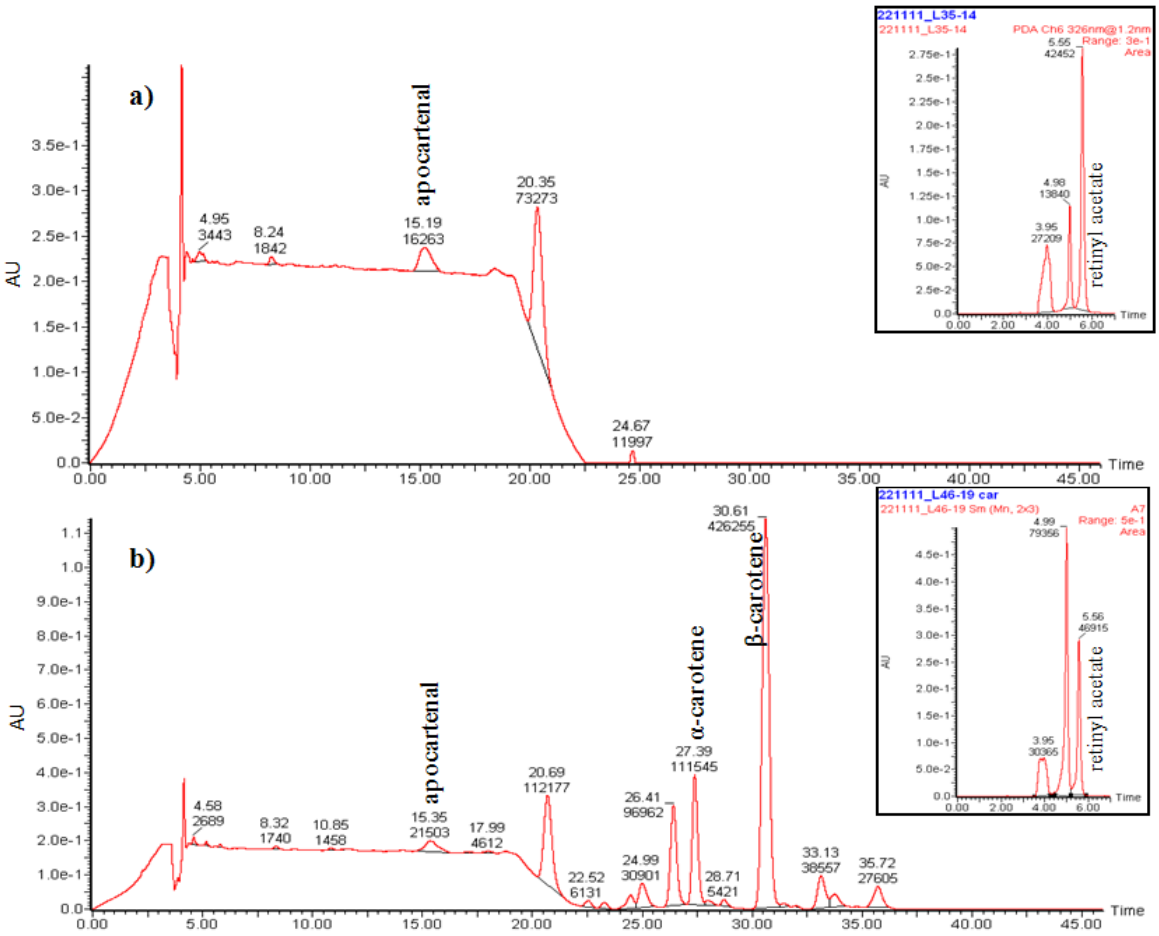

Figure B-3: Sample UPLC-PDA chromatogram for rat liver carotenoids. (a) Sample of D group demonstrating a lack of α-carotene and β-carotene measured at the range of 420-460 nm. Internal standard retinyl acetate measured at 326 nm, 10µl injection. (b) Sample of DC group containing α-carotene and β-carotene measured at the range of 320-385 nm. Internal standard retinyl acetate measured at 326 nm, 10µl injection.

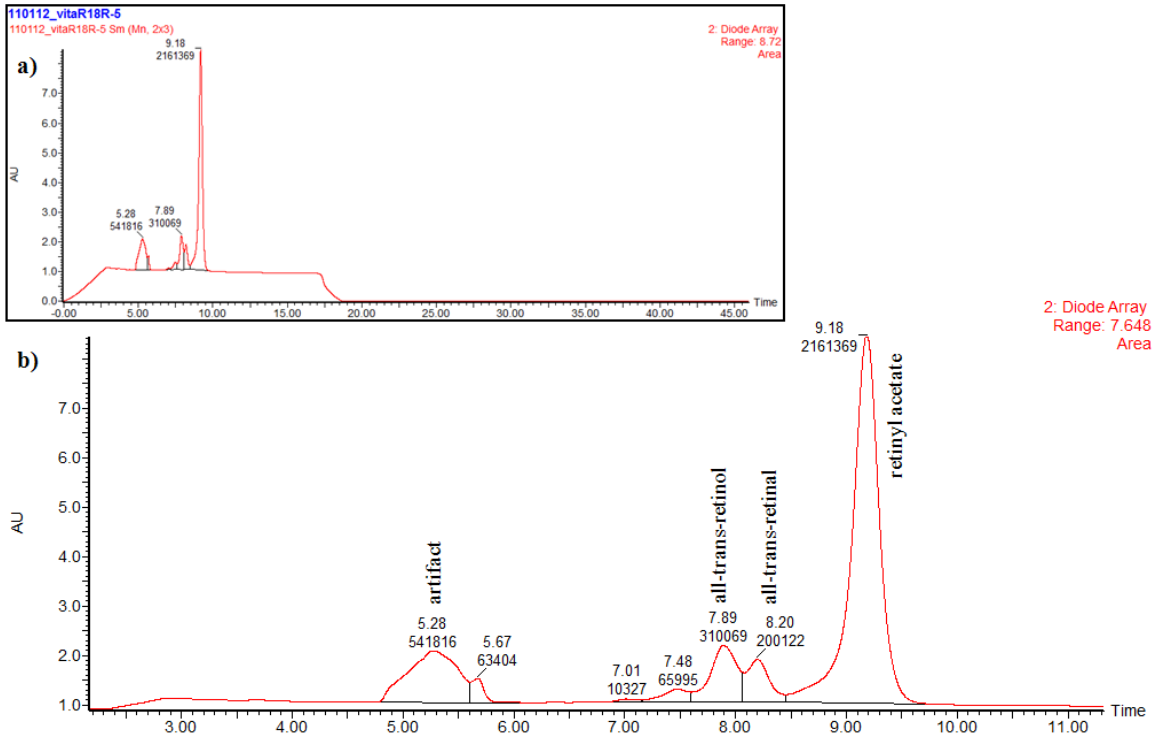

Figure B-4: Sample UPLC-PDA chromatogram for rat retina retinoids. (a) Sample of NC group chromatogram for rat retina containing all-*trans*-retinol, all-*trans*-retinal and internal standard retinyl acetate measured at the range of 320-385 nm. (b) Enhanced view from 0-11 minutes with identification of all-*trans*-retinol, all-*trans*-retinal and internal standard retinyl acetate measured at the range of 320-385 nm, 10µl injection.

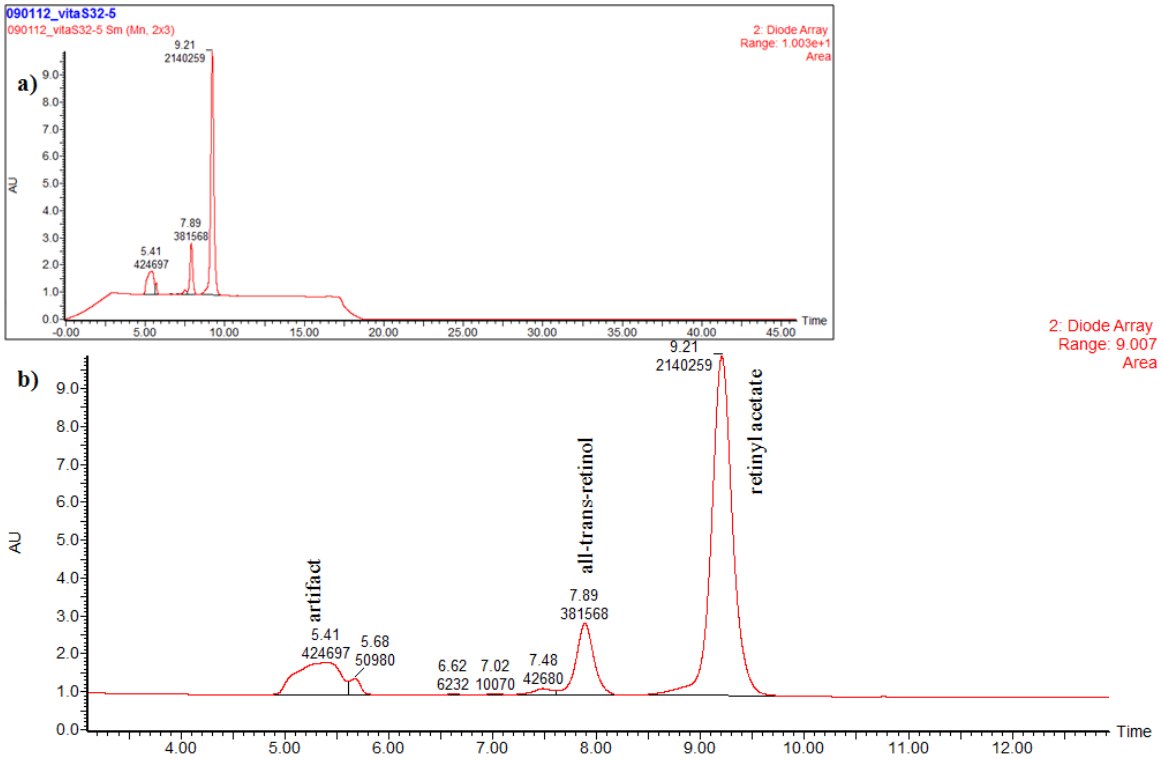

Figure B-5: Sample UPLC-PDA chromatogram for rat serum retinoids. (a) Sample of D group chromatogram for rat serum containing all-*trans*-retinol and internal standard retinyl acetate measured at the range of 320-385 nm. (b) Enhanced view from 0-12 minutes with identification of all-*trans*-retinol and internal standard retinyl acetate measured at the range of 320-385 nm, 10µl injection.

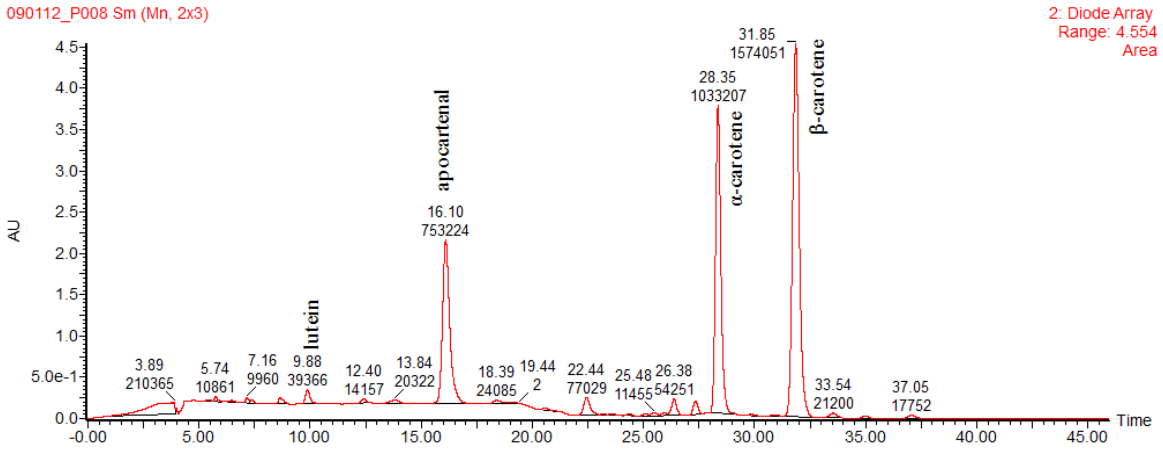

Figure B-6: Sample UPLC-PDA chromatogram for carrot powder taken at 6 months. Contains lutein, internal standard *trans*-β-apo-8'-carotenal, α-carotene and β-carotene measured in the range of 420-460 nm 10µl injection.

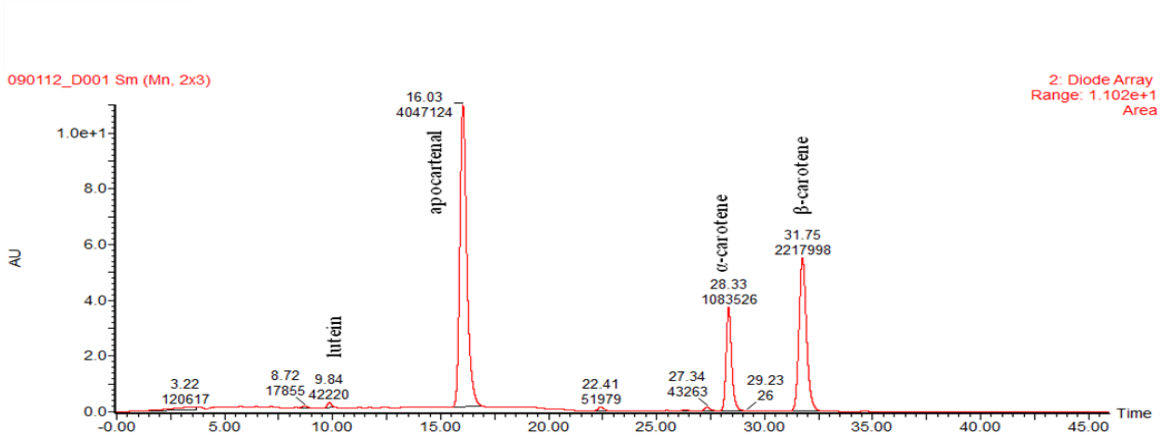

Figure B-7: Sample UPLC-PDA chromatogram for carrot enriched diet taken at 6 months. Contains lutein, internal standard *trans*-β-apo-8'-carotenal, α-carotene and β-carotene measured in the range of 420-460 nm, 10µl injection.