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Retinal Accumulation of Zeaxanthin, Lutein, and β-Carotene in Mice Deficient in Carotenoid Cleavage Enzymes

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Abstract

Carotenoid supplementation can prevent and reduce the risk of age-related macular degeneration (AMD) and other ocular disease, but until now, there has been no validated and well-characterized mouse model which can be employed to investigate the protective mechanism and relevant metabolism of retinal carotenoids. β-Carotene oxygenases 1 and 2 (BCO1 and BCO2) are the only two carotenoid cleavage enzymes found in animals. Mutations of the *bco2* gene may cause accumulation of xanthophyll carotenoids in animal tissues, and BCO1 is involved in regulation of the intestinal absorption of carotenoids. To determine whether or not mice deficient in BCO1 and/or BCO2 can serve as a macular pigment mouse model, we investigated the retinal accumulation of carotenoids in these mice when fed with zeaxanthin, lutein, or β-carotene using an optimized carotenoid feeding method. HPLC analysis revealed that all three carotenoids were detected in sera, livers, retinal pigment epithelium (RPE)/choroids, and retinas of all of the mice, except that no carotenoid was detectable in the retinas of wild type (WT) mice. Significantly higher amounts of zeaxanthin and lutein accumulated in the retinas of BCO2 knockout ($bco2^{-/-}$) mice and BCO1/BCO2 double knockout ($bco1^{-/-}/bco2^{-/-}$) mice relative to BCO1 knockout ($bco1^{-/-}$) mice, while $bco1^{-/-}$ mice preferred to take up β-carotene. The levels of zeaxanthin and lutein were higher than β-carotene levels in the $bco1^{-/-}/bco2^{-/-}$ retina, consistent with preferential uptake of xanthophyll carotenoids by retina. Oxidative metabolites were detected in mice fed with lutein or zeaxanthin but not in mice fed with β-carotene. These results indicate that $bco2^{-/-}$ and $bco1^{-/-}/bco2^{-/-}$ mice could serve as reasonable non-primate models for macular pigment function in the vertebrate eye, while $bco1^{-/-}$ mice may be more useful for studies related to β-carotene.

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Keywords

Knockout mice; carotenoid cleavage; retina; HPLC; carotenoid metabolism

1. Introduction

Lutein, zeaxanthin, and *meso*-zeaxanthin are the macular carotenoids present in the human retina (Beatty et al., 1999; Berendschot et al., 2002; Bernstein et al., 2016). These carotenoids are well-known natural antioxidants and light screening compounds that are capable of quenching singlet oxygen and other free radicals (Krinsky, 1989; Li et al., 2010) and absorbing potentially damaging blue light. Retinal content of these macular carotenoids is inversely associated with the incidence of age-related macular degeneration (AMD), a leading cause of blindness in western countries (Landrum et al., 1997; LaRowe et al., 2008). Dietary carotenoid supplementation can increase macular carotenoid levels in the human retina (Beatty et al., 1999; Bernstein et al., 2016; Nolan et al., 2016), and the recent Age-Related Eye Disease Study 2 (AREDS2) with nearly 5000 subjects reported that daily supplementation with 10 mg of lutein and 2 mg of zeaxanthin is a safe and effective substitute for the β-carotene of the original AREDS formula and can limit and/or slow the progression of AMD (Age-Related Eye Disease Study 2 Research et al., 2013). Additionally, clinical studies have shown that carotenoid supplementation can actually improve visual performance in some subjects (Hammond et al., 2014; Nolan et al., 2016; Sabour-Pickett et al., 2013; Stringham and Hammond, 2008).

Many experiments have been carried out to investigate the ocular effects of carotenoid supplementation using different animal models such as monkey, quail, chicken, rat, and mouse(Bhosale et al., 2007; Fernandez-Robredo et al., 2013; Johnson et al., 2005; Shapiro et al., 1984). Feeding carotenoids can significantly increase the carotenoid levels in the retinas of monkeys and birds; however, it is challenging to deliver carotenoids to the retina of the mouse, a very popular small laboratory animal with a multitude of genetic and environmental models of AMD. Numerous researchers have fed carotenoids to mice in the hope of modifying the course of various eye diseases. In some studies, they looked at the whole eye but have never conclusively demonstrated that administered carotenoids actually reach the retina as opposed to just the retinal pigment epithelium (RPE) and choroid(Gorusupudi and Vallikannan, 2012; Lakshminarayana et al., 2008; Mamatha and Baskaran, 2011), casting doubt on the validity of some of their conclusions. Therefore, we contend that it is necessary to develop validated mouse models which can reproducibly deposit physiologically relevant levels of carotenoid in the retina to facilitate future studies of the role of carotenoids in the prevention and treatment of AMD and other ocular disorders.

β-Carotene oxygenases 1 and 2 (BCO1 and BCO2) are the only two carotenoid cleavage enzymes found in animals. BCO1, also known as β, β-carotene 15, 15′-monooxygenase, mainly cleaves provitamin A carotenoids such as β-carotene or β-cryptoxanthin at the 15, 15′ carbon-carbon double bond to yield all-*trans*-retinal, all-*trans*-3-hydroxy retinal (in the case of β-cryptoxanthin), or acyloretinal (in the case of lycopene) (Bhatti et al., 2003; dela

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Sena et al., 2013; Lindqvist and Andersson, 2002; Mein et al., 2011). All-*trans*-retinal can be converted to all-*trans*-retinol which participates in the visual cycle as well as a variety biological processes in other tissues (Krinsky et al., 1993), or it can be oxidized to retinoic acid which is critical for various gene regulation pathways. BCO2 was previously called β, β-carotene 9′, 10′-dioxygenase, and it splits carotenoids, especially xanthophyll carotenoids, eccentrically at the 9′, 10′ double-carbon bond and yields apo-10′-carotenoid and ionone, or rosafluene (Amengual et al., 2011; Dela Sena et al., 2016; Mein et al., 2011). Nonsense mutations or single nucleotide polymorphisms (SNPs) in the promoter region of the *bco2* gene may cause an accumulation of lutein or zeaxanthin in animals, turning their skin, meat, and fat a yellowish color (Berry et al., 2009; Vage and Boman, 2010).

The human macula is a yellow spot rich in carotenoids, and we and others have recently shown that human retinal BCO2 is a relatively inactive carotenoid cleavage enzyme in contrast to the very active cleavage enzymes found in most non-primate vertebrates such as chickens and mice (Li et al., 2014, Dela Sena et al., 2016). Human BCO2's poor carotenoid cleavage activity appears to be important in facilitating carotenoid deposition in human ocular tissues (Li et al., 2014). Although BCO1 does not cleave lutein or zeaxanthin in vertebrates, it still can indirectly influence their tissue levels through regulation of scavenger receptor BI (SR-BI), the nonspecific carotenoid transporter in the intestine, as evidenced by significant association of BCO1's genetic variants with serum carotenoid levels and macular pigment optical density (MPOD) (Borel et al., 2011; Meyers et al., 2013).

In this study, we take advantage of recent insights into the key role of BCO enzymes in regulating ocular carotenoid concentrations to engineer and characterize "macular pigment mice" enriched in retinal carotenoids that could be utilized to study the protective effects of lutein, zeaxanthin, and β-carotene in genetic and environmental models of human ocular disease.

2. Materials and Methods

2.1 Materials

Carotenoid standards lutein and zeaxanthin were provided by Kemin Health (Des Moines, IA, USA) and DSM Nutritional Products, Ltd. (Kaiseraugst, Switzerland), respectively. The β-carotene standard was purchased from Sigma Chemicals (Saint Louis, MO, USA). All carotenoid standards were crystalline with > 98% purity confirmed by HPLC. Arabinogalactan (AG) and β-glycyrrhizic acid (GA) were purchased from Sigma Chemicals. Captisol® was obtained from Ligand Pharmaceuticals, Inc. (San Diego, CA, USA). Sucrose monolaurate (SML) was provided by Mitsubishi Chemicals (Tokyo, Japan). Lutein, zeaxanthin, and β-carotene ActiLease® beadlets were supplied by DSM Nutritional Products, Ltd. (Kaiseraugst, Switzerland). All organic solvents used were of HPLC grade and were purchased from Thermo Fisher (Waltham, MA, USA).

2.2 Carotenoid formulations for feeding method optimization

Zeaxanthin was chosen to study carotenoid uptake in wild type (WT) mice. We tested six different methods to optimize its uptake and bioavailability, which included Captisol®, SML,

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crystalline zeaxanthin, β-glycyrrhizic acid, arabinogalactan, and DSM ActiLease® beadlets. Captisol® is a polyanionic β-cyclodextrin derivative with a sodium sulphonate salt which was used to solubilize zeaxanthin. The Captisol®-carotenoid complex was prepared by grinding 50 mg zeaxanthin with 1 g Captisol® using a pestle and mortar and adding a few drops of dimethyl sulfoxide (DMSO)/water (95: 5 v/v). After mixing the complex, a 5% zeaxanthin stock solution was prepared in water containing 0.01% ascorbic acid. Zeaxanthin-SML was prepared as previously described (Fullmer and Emmick, 2005) with slight modifications. First, zeaxanthin (0.5 g) was mixed with a saturated solution of 30 g sucrose monolaurate in 150 ml of water followed by a 10-minute centrifugation at 2,400 x g. Insoluble zeaxanthin was filtered out using a 0.2 μm nylon filter. The final carotenoid concentration was 14 μg/mL. Preparations of water-soluble complexes with AG and GA (1 g/kg) were made by the mechanochemical method described in detail elsewhere (Dushkin et al., 2013; Polyakov et al., 2009). For the crystalline zeaxanthin bioavailability study, zeaxanthin was mixed with the vitamin-A-deficient AIN-93G diet (1 g/kg) prepared by Test Diet (St. Louis, MO, USA). Zeaxanthin encapsulated DSM ActiLease® beadlets were mixed with a previously reported base diet (Lindshield et al., 2008) at a dosage of 1 g/kg of the base diet.

2.3 Animal handling and carotenoid feeding experiments

Eight to ten -week-old *bco1*$^{-/-}$, *bco2*$^{-/-}$, and BCO1/BCO2 double knockout (*bco1*$^{-/-}$/*bco2*$^{-/-}$) mice were bred in the University of Utah vivarium using founders from Case Western Reserve University (gifts from Dr. Johannes von Lintig). C57BL/6 WT mice were purchased from Jackson Laboratories (Farmington, CT, USA). Mice were housed in the vivarium under controlled conditions (26°C, 12-h light/dark cycle) with *ad libitum* access to food and water. The University of Utah Institutional Animal Care and Use Committee approved all animal procedures. For the optimization of the carotenoid formulations, twelve-week-old C57BL/6 WT mice (n=5–10 mice/carotenoid feeding group) were used. The carotenoids were delivered either by daily gavage, dissolving in drinking water, or by incorporating into the diet. After optimizing the feeding method, mice were put on an AIN-93G vitamin-A-deficient diet for four weeks, after which the experimental groups (n=18–25 mice/genotype, 4 genotypes in each carotenoid feeding group) received zeaxanthin, lutein, or β-carotene in DSM ActiLease® beadlets (DSM Nutritional Products, Basel, Switzerland) mixed into the base diet (1 g/kg) for another four weeks. The control group received placebo beadlets mixed with the base diet. At the end of the study, the mice were anesthetized with isoflurane (1–3%), and blood was collected after decapitation. Retina, RPE/choroid, serum, and liver samples were collected and stored at −80°C.

2.4 Carotenoid extraction and high performance liquid chromatography (HPLC) analysis

Carotenoids in the liver, serum, and ocular tissues were extracted and analyzed as described before with slight modifications (Khachik et al., 2002). Briefly, pooled ocular tissues (n=3–5 pairs) were homogenized using a Bead Beater (BioSpec, Bartlesville, OK, USA), and the carotenoids were extracted using tetrahydrofuran (THF) containing 0.1% butylated hydroxytoluene (BHT). The liver samples were first homogenized with a mechanical homogenizer (PRO Scientific, Oxford, CT, USA), and carotenoids were extracted from 50–300 mg of the homogenized liver using the same method used for ocular tissues. Serum

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carotenoids were extracted from a 0.2 mL serum sample using ethanol: ethyl acetate (4:10) followed by 1 mL hexane extraction. All extraction procedures were repeated three to four times. The pooled extracts of all tissues were evaporated to dryness under nitrogen gas at room temperature. The dried samples were re-dissolved in 0.2 mL HPLC mobile phase and centrifuged at 13,000 x g for 15 minutes, and the supernatants were injected onto a silica-based nitrile-bonded column (25 cm length × 4.6 mm internal diameter, 5-μm spherical particle size; Regis Chemical, Morton Grove, IL, USA) for lutein and zeaxanthin HPLC analysis. The mobile phase consisted of an isocratic mixture of hexanes (75%), dichloromethane (25%), methanol (0.3%), and N, N-diisopropylethylamine (DIPEA, 0.1%) with a flow rate of 1 mL/min. For the β-carotene analysis, HPLC separation was conducted at a flow rate of 1.0 mL/min on a C30 column (25 cm length × 4.6 mm internal diameter; YMC Carotenoids Allentown, PA, USA) with a linear gradient of methanol and methylene chloride (% methanol at min: 99 at 0; 90 at 10; 70 at 20; 0 at 30; 99 at 35; 99 at 40). The columns were maintained at room temperature, and the HPLC detector was monitored at 450 nm. Carotenoid peak identities were confirmed by retention time, photodiode array (PDA) spectra, and co-elution with authentic standards.

2.5 Statistical analysis

Carotenoid contents of serum, liver, and the ocular tissues of the mice were analyzed using ANOVA and *t*-tests. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). The bars on the graphs represent means with ± SD. $P < 0.05$ was considered significant.

3. Results

3.1 Optimization of carotenoid feeding method

In order to optimize and standardize the carotenoid feeding method for subsequent experiments, six food-grade carotenoid solubilization and oral delivery methods were evaluated by comparing relative bioavailability of zeaxanthin in WT mice (Figure 1). The experimental groups (n=5–10) received zeaxanthin in Captisol® (~12.5 mg/day/mouse) by daily gavage, zeaxanthin in SML (~0.14 mg/day/mouse) in drinking water, crystalline zeaxanthin mixed with normal mouse diet, zeaxanthin mixed in GA or AG in vitamin-A-deficient diet, or zeaxanthin in DSM beadlet diet. All carotenoid diet fed mice consumed ~2.6 mg of carotenoid per day. Mice fed placebo DSM beadlets served as the no-carotenoid control.

After feeding zeaxanthin for four weeks, all six methods increased the zeaxanthin level in the serum of WT mice to varying degrees relative to the undetectable levels in the control mice. DSM beadlets proved to be the best delivery method, with serum zeaxanthin reaching 460 ng/mL, which was around 10 to 200 times higher than the other five methods. It is also much higher than a typical zeaxanthin level in human serum (~93 ng/mL). A similar pattern was seen in the accumulation of zeaxanthin in liver, where DSM beadlet supplementation was again the most effective method, raising the level of zeaxanthin in liver to 1572 ng/g. Interestingly, only the GA, AG, and DSM beadlet methods are able to successfully deliver zeaxanthin into the RPE/choroid of WT mice. Around 0.81 ng zeaxanthin was detected per

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pair of RPE/choroid in WT mice fed with DSM beadlet zeaxanthin diet, which is about 3 to 5 times higher than the amount found in mice fed with GA- or AG-zeaxanthin. No zeaxanthin was detected in the retinas of mice fed zeaxanthin using any one of these six methods. Our results show that DSM beadlet carotenoids mixed into diet is the most effective of these six carotenoid delivery methods, and we therefore chose to use this method for all subsequently reported experiments in transgenic mice.

3.2 Zeaxanthin feeding experiments

Dietary zeaxanthin accounts for one-third of the carotenoids that accumulate in the human macula. We have previously reported the accumulation of zeaxanthin in the ocular tissues of *bco2*$^{-/-}$ mice (Li et al., 2014). Here, we systematically investigated the accumulation of zeaxanthin in sera, livers, RPE/choroids, and retinas of *bco1*$^{-/-}$, *bco2*$^{-/-}$, BCO1/BCO2 double knockout (*bco1*$^{-/-}$/*bco2*$^{-/-}$), and WT mice (Figure 2). There was 460.6 ng/mL zeaxanthin in the serum of WT mice, and knocking out the *bco1* gene caused no change to the serum levels of zeaxanthin relative to WT mice. In comparison with that of WT mice, the serum zeaxanthin level was significantly increased in both *bco2*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice, while no significant difference was found between *bco2*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice. The profile of hepatic zeaxanthin in the four groups of mice demonstrated a similar pattern to their serum zeaxanthin. Zeaxanthin contents in the RPE/choroid of WT, *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice were 0.80, 0.58, 3.26 and 4.73 ng/pair, respectively. Statistical analysis revealed that the zeaxanthin content in the RPE/choroids of *bco2*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice was significantly higher in comparison to that of WT mice, and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice had significantly higher RPE/choroid levels than single knockouts. Retinal zeaxanthin was found in *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice but not in WT mice. There were 0.71 ng zeaxanthin in the retina (per pair) of *bco2*$^{-/-}$ mice, which is close to our previous results (Li et al., 2014). The *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice had significantly increased retinal zeaxanthin levels relative to single knockouts. Comparable *meso*-zeaxanthin feeding experiments were not performed because there is no available beadlet formulation containing *meso*-zeaxanthin.

3.3 Lutein feeding experiments

Lutein is another dietary carotenoid rich in the human macula. Quail and monkey feeding experiments have indicated that lutein is the precursor of *meso*-zeaxanthin in the human retina (Bhosale et al., 2007; Johnson et al., 2005). Our lutein feeding experiment demonstrated that the pattern of lutein accumulation in all the tissues of *bco1*$^{-/-}$, *bco2*$^{-/-}$, *bco1*$^{-/-}$/*bco2*$^{-/-}$, and WT mice is similar to that of the zeaxanthin feeding experiments with the exception of slightly lower levels of tissue carotenoids (Figure 3). The serum lutein levels were 388, 486, 943 and 1107 ng/mL in WT, *bco1*$^{-/-}$, *bco2*$^{-/-}$, *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice, respectively. HPLC analysis showed that the hepatic lutein content was around 1675 ng/g in WT mice, 1678 ng/g in *bco1*$^{-/-}$ mice, 2777 ng/g in *bco2*$^{-/-}$ mice, and 2521 ng/g in *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice. Lutein levels in the RPE/choroids of WT, *bco1*$^{-/-}$, *bco2*$^{-/-}$, *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice were 0.37, 0.38, 2.07 and 3.24 ng/pair, respectively. No lutein was detected in the retina of WT mice. After supplementation with lutein, the retinal lutein levels were elevated to 0.03 ng/pair in *bco1*$^{-/-}$ mice, 0.55 ng/pair in *bco2*$^{-/-}$ mice, and 0.89 ng/pair in *bco1*$^{-/-}$/

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bco2$^{-/-}$ mice. In these lutein feeding experiments, no *meso*-zeaxanthin was ever detected in any tissue of the mice when we checked with chiral chromatography.

3.4 β-Carotene feeding experiments

Given that β-carotene is a pro-vitamin A carotenoid, we also carried out a β-carotene feeding experiment even though β-carotene is not detectable in the human macula. Figure 4 shows that the serum β-carotene level was 609 ng/mL in WT mice. Knocking out BCO1 significantly increased β-carotene to 1659 ng/mL in the sera of *bco1*$^{-/-}$ mice, while knocking out BCO2 did not significantly change the serum β-carotene of *bco2*$^{-/-}$ mice relative to WT mice. β-Carotene in the serum of *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice was around 2.3 times higher than that of *bco1*$^{-/-}$ mice. The accumulation pattern of β-carotene in liver is similar to that of serum. It also showed that the levels of hepatic β-carotene were around 10 to 40 times more than the hepatic lutein or zeaxanthin in all four genotyped mouse groups. β-Carotene levels in RPE/choroid of *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice were significantly higher than in WT mice, while no significant difference was found between *bco1*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice. No β-carotene was detected in the retina of WT mice. The pattern of β-carotene accumulation in the retina of *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice were similar to that of the RPE/choroids, but their carotenoid levels were much lower than those in the lutein or zeaxanthin feeding experiments.

3.5 The metabolites of zeaxanthin and lutein in the tissues of mice

Oxidative metabolites of zeaxanthin and lutein in the eyes and other tissues of mice have been reported by other groups and have been identified as likely to include di-dehydrozeaxanthin and di-dehydrolutein based on PDA spectra and mass spectral analyses (Amengual et al., 2011). We observed similar metabolites in our chromatograms with xanthophyll-like PDA spectra, but since we did not have authentic standards or mass spectrometry, we choose to report their levels in aggregate. In our carotenoid feeding experiments, oxidative metabolites were found in the tissues of mice in the zeaxanthin and lutein groups, while there were no detectable carotenoid oxidative metabolites detected in the β-carotene group (Figures S1–3). Figures S1 and S2 show that the metabolites of lutein and zeaxanthin were detected in the retinas of *bco2*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice but not in the *bco1*$^{-/-}$ or WT mice. The concentration of zeaxanthin is around 2 to 3 times more than that of zeaxanthin metabolites in the RPE/choroids of *bco2*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice, while this ratio is almost reversed in their retinas. Interestingly, the amounts of lutein were much lower than its metabolites in the RPE/choroids and retina of *bco2*$^{-/-}$ mice. The content of lutein is around 3 times higher than its metabolites in the RPE/choroids and is nearly equal to its metabolites in the retina of *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice. More metabolites of lutein and zeaxanthin than themselves were detected in the serum and liver of *bco2*$^{-/-}$ and *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice; whereas, in WT and *bco1*$^{-/-}$ mice, the contents of lutein and zeaxanthin in the serum and liver were higher than their metabolites (Figures S1 and S2). No β-carotene metabolites with carotenoid-like PDA spectra were detected in any tissues of the mice in the β-carotene feeding experiment (Figure S3), although the contents of β-carotene in relevant tissues were comparable to xanthophyll contents in lutein and zeaxanthin feeding experiments.

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4. Discussion

Crystalline carotenoids are lipophilic compounds with poor oral bioavailability, especially in mouse models. To optimize bioavailability, we tried zeaxanthin in six different formulations such as crystalline zeaxanthin in the diet, Captisol®, SML, AG, GA, and DSM ActiLease® beadlets. Captisol® and SML were given orally, and the rest were fed by incorporating them into the diet. The rationale for choosing these formulations was based on previous studies that showed enhanced solubility and stability of carotenoids in these complexes (Apanasenko et al., 2015; Lockwood et al., 2003). Captisol® is a well-characterized cyclodextrin derivative that has been used in pharmaceutical industries to improve the solubility of hydrophobic drugs. Sucrose fatty acid esters (SML) are FDA-approved solubilization agents for the dispersion of carotenoids in clear beverages. Although we achieved better solubility of carotenoids in the Captisol® and SML formulations, their serum bioavailability was very similar to the crystalline zeaxanthin, but in the liver, crystalline zeaxanthin was slightly better.

We further explored ways to improve the bioavailability of carotenoids. Accordingly, we studied GA, AG, and DSM ActiLease® beadlets for carotenoid stabilization and delivery. These nanosized complexes have better solubility and higher bioavailability when they are used in food processing as well as in the production of therapeutic formulations (Apanasenko et al., 2015; Polyakov & Kispert, 2015; Polyakov et al., 2013). GA (also known as glycerrhizin) belongs to the triterpene glycosides extracted from the licorice root (*Glycyrrhiza glabra L*) and contains both hydrophilic (glucuronic acid) and hydrophobic (glycyrrheretic acid) regions. It was suggested in many studies that GA in a solution can create cyclic structures that can form inclusion complexes with various organic compounds as well as micelles at high concentrations. AG is a natural polysaccharide, a branched polymer with a molecular mass of 15–20 kDa. It is found in a variety of plants but is more abundant in the *Larix* genus, primarily the Western and Siberian larch. The increased solubility of AG-zeaxanthin complex is due to the hydrophilic nature of AG. Even though nanosized AG and GA carotenoid complexes improved the solubility and bioavailability, we did not see any retinal uptake of zeaxanthin in the WT mice. Finally, we evaluated the DSM ActiLease® beadlets, which is a patented carotenoid formulation prepared by DSM Nutritional Products Ltd but there was still no retinal uptake. Out of the six carotenoid formulations that we evaluated, we learned that mixing DSM carotenoid beadlets in the diet was the best way to improve general bioavailability of the carotenoid in the WT mouse. Therefore, the DSM ActiLease® beadlet diet was chosen for the subsequent carotenoid feeding experiments in transgenic mice in which carotenoid cleavage enzymes (BCO1 and/or BCO2) had been knocked out to enhance carotenoid delivery to the retina.

Our carotenoid feeding experiments demonstrated that in comparison with WT mice, the concentrations of lutein and zeaxanthin were significantly elevated in all of the tested tissues in $bco2^{-/-}$ and $bco1^{-/-}/bco2^{-/-}$ but not $bco1^{-/-}$ mice, while the accumulation of β-carotene was dramatically increased in the $bco1^{-/-}$ and $bco1^{-/-}/bco2^{-/-}$ but not $bco2^{-/-}$ mice. This is consistent with the relevant findings from other groups (Amengual et al., 2013; dela Sena et al., 2013; Dela Sena et al., 2016; Lindqvist and Andersson, 2002; Lindshield et al., 2008; Mein et al., 2011; Wang, 2012). Our feeding data also revealed that in $bco1^{-/-}/bco2^{-/-}$ mice,

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the retinal accumulation of xanthophylls lutein and zeaxanthin is around six times higher than retinal accumulation of β-carotene, while the hepatic β-carotene is around twenty times as much as the hepatic xanthophyll. This preferential absorption of xanthophyll carotenoids over β-carotene by retina has also been shown by others in *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice fed with a diet containing a mixture of β-carotene and zeaxanthin with a ratio of 1 to 3, and this ratio changed to 1:2 in the liver of mice (Palczewski et al., 2014). These results suggest that certain carotenoids can be selectively absorbed by tissues, consistent with our previous work on selective carotenoid binding proteins such as steroidogenic acute regulatory domain protein 3 (StARD3) and glutathione S-transferase Pi isoform (GSTP1) (Bhosale et al., 2004; Li et al., 2011).

In addition, compared with *bco2*$^{-/-}$ mice, zeaxanthin levels increased 1.4 and 1.6 times in the RPE/choroid and retina of *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice. Likewise, lutein levels are elevated 2.3 times in the RPE/choroid, and 1.6 times in the retina of *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice relative to *bco2*$^{-/-}$ mice (Figure 2 and 3). These feeding data imply that BCO1 may regulate the xanthophyll accumulation in the mouse eye. Meanwhile, our β-carotene feeding experiments showed that β-carotene levels in the liver of *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice were significantly elevated to twice the level found in *bco1*$^{-/-}$ mice, which is similar to findings from another group (Amengual et al., 2013). This suggests that BCO2 may modulate hepatic β-carotene accumulation.

Several carotenoid metabolites have been detected in BCO knockout mice fed with lutein and zeaxanthin (Amengual et al., 2011; Babino et al., 2015). The major metabolites were identified as likely to include di-dehydrolutein and di-dehydrozeaxanthin through mass spectrometry. Although we could not definitively identify these carotenoid metabolites in our experiments due to a lack of relevant standards and no mass spectrometry, we observed metabolites with similar PDA spectra, but with somewhat different retention patterns and times. We suspect that the lutein and zeaxanthin metabolites are initially formed in the gut because they were also observed in the intestinal contents at the time of necropsy. On the other hand, unlike the lutein and zeaxanthin feeding experiments, we did not detect any oxidation products of carotenoids in the β-carotene feeding experiments. In addition, we did not detect *meso*-zeaxanthin in the retina or RPE/choroid of mice fed with lutein, a result in contrast to the demonstrated ability of birds and primates to convert lutein into *meso*-zeaxanthin in ocular tissues (Bhosale et al., 2007; Gorusupudi et al., 2016; Johnson et al., 2005).

Our work has shown that mice deficient in one or two carotenoid cleavage enzyme can accumulate carotenoids in their retinas. The *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice are able to accumulate the highest amounts of carotenoids in their tissues, and they therefore may be used to evaluate the bioavailability of many different carotenoids; however, given that *bco2*$^{-/-}$ mice are capable of accumulating comparable amounts of xanthophyll as the *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice in their retinas, and breeding single knockout mice can save considerable time, *bco2*$^{-/-}$ mice may be the most useful mouse model used to investigate the uptake, transport, and metabolism of the macular carotenoids. The *bco1*$^{-/-}$ mouse seems unlikely to be a good model for most retinal carotenoid studies due to their poor retinal accumulation of

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xanthophylls, but they may serve as an excellent model for β-carotene studies, especially in tissues such as liver and serum.

Mice have been one of the most commonly employed laboratory animal models for carotenoid uptake and metabolism. Compared with other non-human primate models, mice are highly cost-effective regarding maintenance and experimental procedures. The availability of their full genomic sequence also makes them an ideal choice for further studying the effect of various genes on carotenoid uptake and metabolism. In our feeding experiments of lutein and zeaxanthin, besides these carotenoids, some carotenoid metabolites are also found in tissues of the tested mice, while there are more metabolites in the BCO knockout mice than WT mice. These metabolic transformations of ingested carotenoids initially happen in the intestine, and they are likely to be subsequently distributed into various tissues. It is unknown whether these carotenoid metabolites have antioxidant properties; however, we assume that they may protect tissues against oxidative damage just like their parent compounds, lutein and zeaxanthin, based on their similar PDA spectra and proposed chemical structures. In addition, we need to supplement a high dosage of carotenoid to our transgenic mice, ~2.6 mg of carotenoid per day, to deliver detectable carotenoid into their retina. This is equivalent to a daily dose of ~634 mg of carotenoid per day for a human weighing 60 kg. It should also be noted that the *fovea centralis* is unique to primates, so macular pigment mice could never fully recapitulate the anatomy and function of the human *macula lutea*. With consideration of these limitations, we believe that BCO2 knockout mice will offer unique value in research to prevent human eye diseases using carotenoids.

5. Conclusions

Our carotenoid feeding experiments demonstrate that mice deficient in BCO1, BCO2, or both BCO1 and BCO2 can reproducibly accumulate lutein and zeaxanthin in their retinas at physiologically relevant levels. Based on their excellent capacity to accumulate xanthophylls in their retinas and their shorter breeding time, the *bco2*$^{-/-}$ mouse appears to be the most suitable macular pigment mouse model. This novel macular pigment mouse model will greatly facilitate our understanding on the physiological roles of the macular carotenoids and their relevant molecular mechanisms of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AG	Arabinogalactan
AMD	Age-related macular degeneration
AREDS2	Age-Related Eye Disease Study 2
BCO1	β-carotene oxygenase 1
BCO2	β-carotene oxygenase 2
BHT	Butylated hydroxytoluene
DIPEA - N	N-diisopropylethylamine
DMSO	Dimethyl sulfoxide
GA- β	glycyrrhizic acid
GSTP1	Glutathione S-transferase Pi isoform
HPLC	High performance liquid chromatography
MPOD	Macular pigment optical density
PDA	photodiode array
RPE	Retinal pigment epithelium
SML	Sucrose monolaurate
SNP	Single nucleotide polymorphism
SR-BI	Scavenger receptor protein BI
StARD3	Steroidogenic acute regulatory domain protein 3
THF	Tetrahydrofuran
WT	Wild type

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Highlights

- BCO1 and BCO2 regulate carotenoid delivery into the mouse retina
- Zeaxanthin and lutein are preferentially accumulated in the mouse retina
- *Bco2*$^{-/-}$ mice can serve as “macular pigment mice” in study of eye disease prevention

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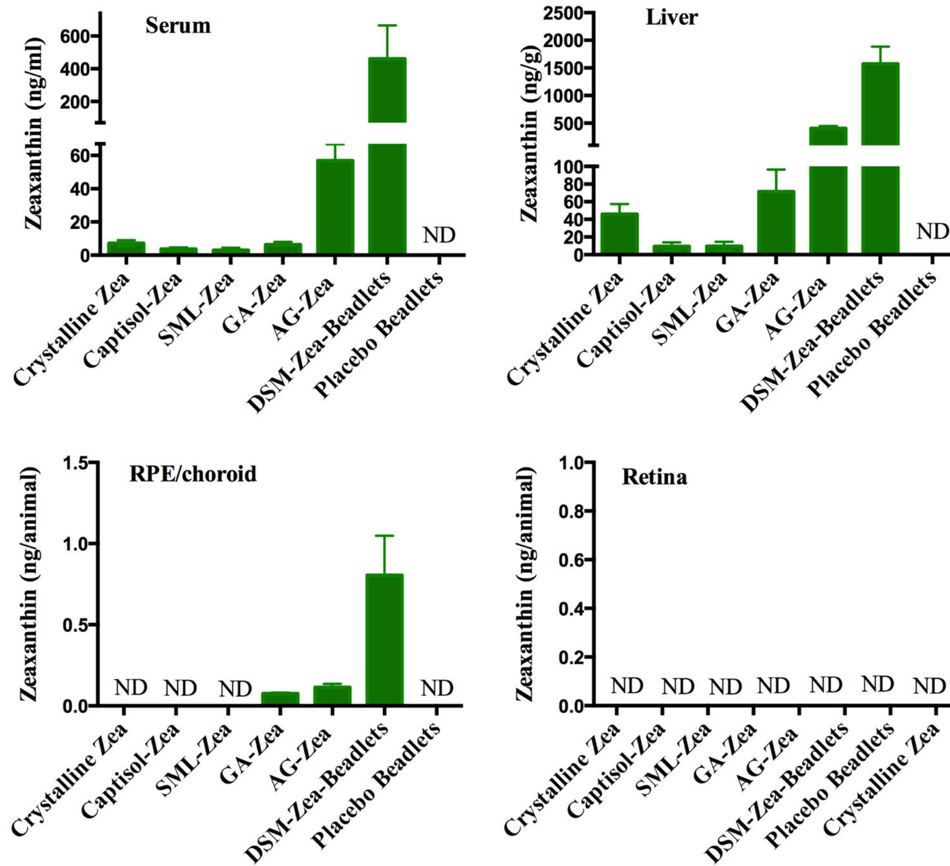

Figure 1. Comparison of six carotenoid delivery methods in C57BL/6 wild type (WT) mice

Twelve-week-old WT mice were fed with zeaxanthin in the formulations of Captisol®, sucrose monolaurate (SML), crystalline zeaxanthin, β-glycyrrhizic acid (GA), arabinogalactan (AG), DSM ActiLease® beadlets, or placebo diet. The zeaxanthin levels in sera, livers, RPE/choroids and retinas of mice were analyzed by HPLC. Values indicate means ± SD from 5 to 10 mice in each group. ND, not detectable.

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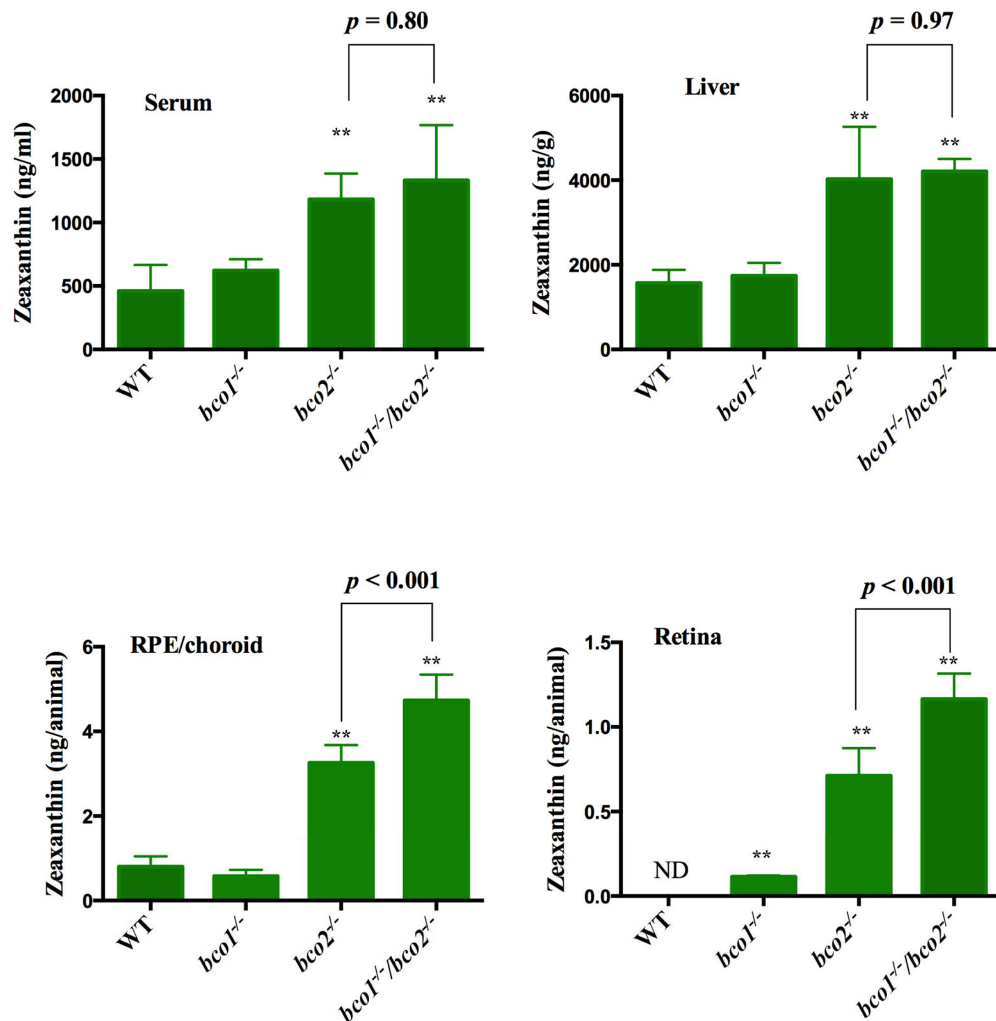

Figure 2. Contents of zeaxanthin detected in the tissues of WT, *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1–/–/ bco2*$^{-/-}$ mice

Twenty-four WT, twenty-two *bco1*$^{-/-}$, twenty-five *bco2*$^{-/-}$ and twenty-three *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice aged from 8 to 10 weeks were kept on DSM zeaxanthin beadlet diet (1 g zeaxanthin/kg diet) for 4 weeks. Carotenoids were extracted from the serum and liver of each individual animal. Retina and RPE/choroid were pooled from 4 to 5 animals (5 repeats) in each mouse group. Values indicate means ± SD. ND, not detectable. *, p<0.05; **, p<0.001.

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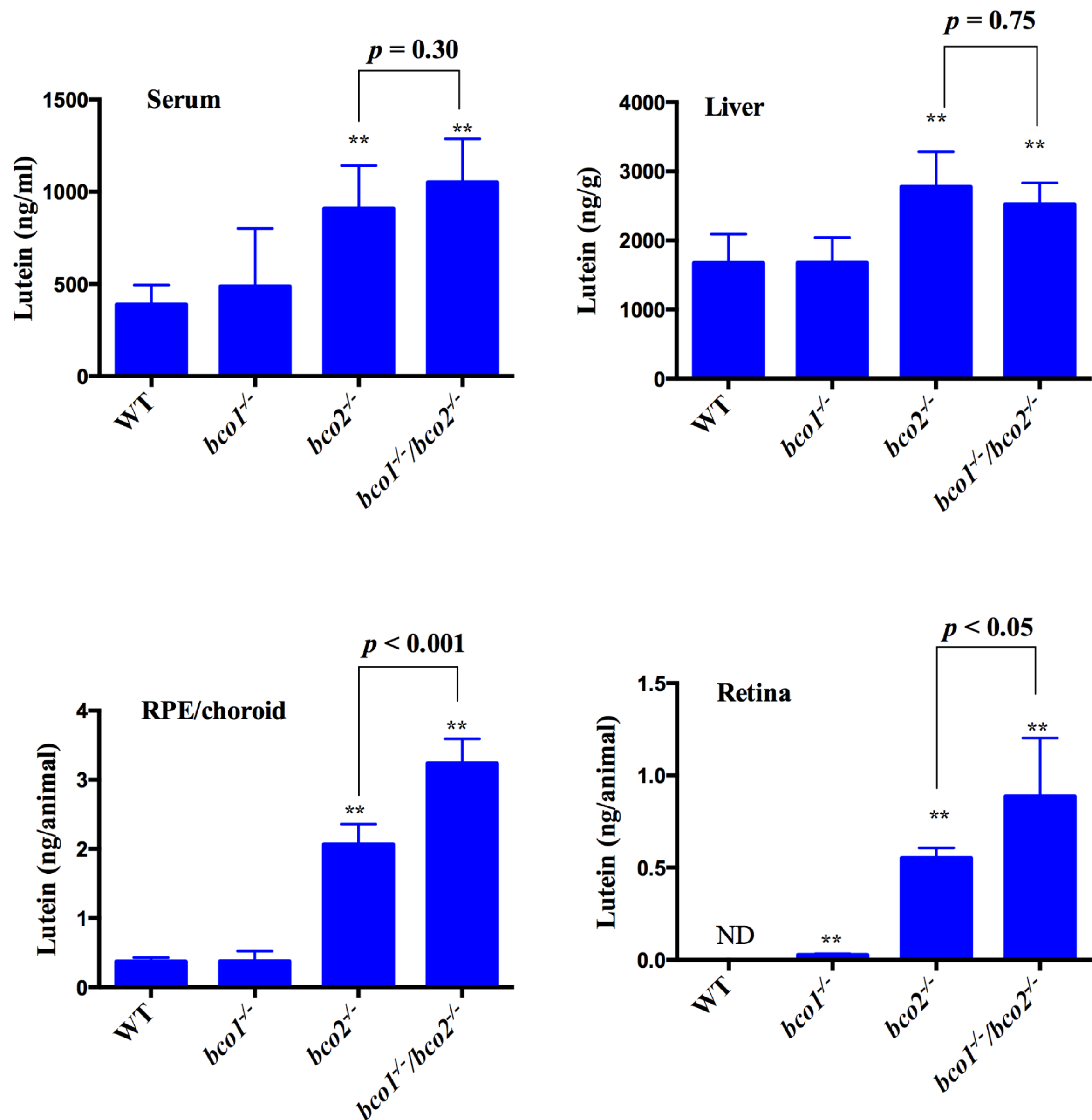

Figure 3. Contents of lutein detected in the tissues of WT, *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1*−/−/*bco2*$^{-/-}$ mice

Twenty-two WT, nineteen *bco1*$^{-/-}$, twenty-one *bco2*$^{-/-}$ and twenty-four *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice aged from 8 to 10 weeks were kept on DSM lutein beadlet diet (1 g lutein/kg diet) for 4 weeks. Carotenoids were extracted from the serum and liver of each individual animal. Retina and RPE/choroid were pooled from 4 to 5 animals (5 repeats) in each mouse group. Values indicate means ± SD. ND, not detectable. *, p<0.05; **, p<0.001.

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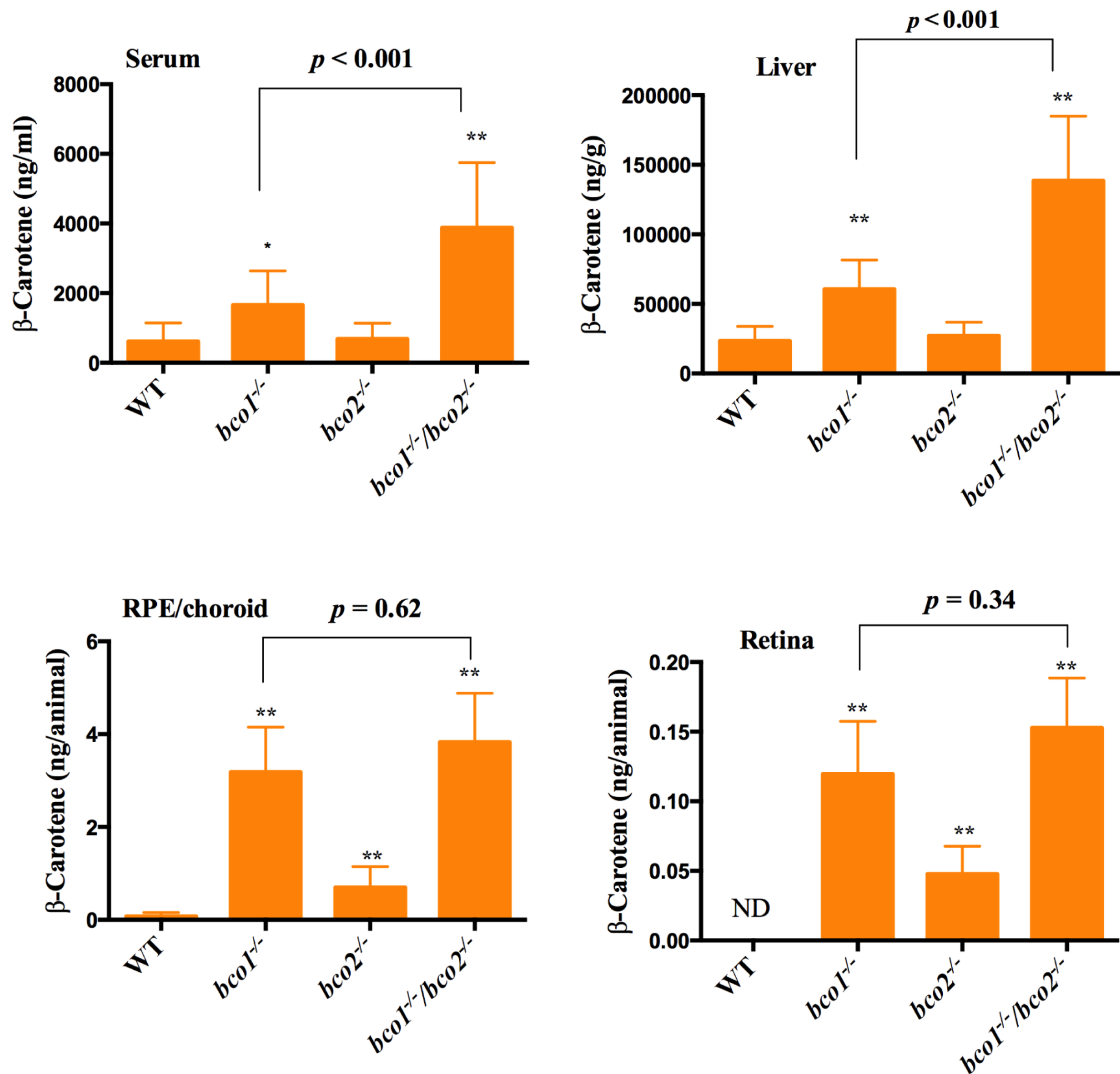

Figure 4. Contents of β-carotene detected in the tissues of WT, *bco1*$^{-/-}$, *bco2*$^{-/-}$, and *bco1*–/–/*bco2*$^{-/-}$ mice

Twenty-three WT, twenty *bco1*$^{-/-}$, twenty *bco2*$^{-/-}$ and eighteen *bco1*$^{-/-}$/*bco2*$^{-/-}$ mice aged from 8 to 10 weeks were kept on DSM β-carotene beadlet diet (1 g β-carotene/kg diet) for 4 weeks. Carotenoids were extracted from the serum and liver of each individual animal. Retina and RPE/choroid were pooled from 3 to 5 animals (5 repeats) in each mouse group. Values indicate means ± SD. ND, not detectable. *, p<0.05; **, p<0.001.