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Supplementation with Macular Carotenoids Improves Visual Performance of Transgenic Mice

Binxing Li[1], **Gregory T. Rognon**[1], **Ty Mattinson**[1], **Preejith P. Vachali**[1], **Aruna Gorusupudi**[1], **Fu-Yen Chang**[1], **Arunkumar Ranganathan**[1], **Kelly Nelson**[1], **Evan W. George**[1], **Jeanne M. Frederick**[1], and **Paul S. Bernstein**[1,*]

[1]Department of Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah School of Medicine, 65 Mario Capecchi Drive, Salt Lake City, Utah, 84132, USA

Abstract

Carotenoid supplementation can improve human visual performance, but there is still no validated rodent model to test their effects on visual function in laboratory animals. We recently showed that mice deficient in β-carotene oxygenase 2 (BCO2) and/or β-carotene oxygenase 1 (BCO1) enzymes can accumulate carotenoids in their retinas, allowing us to investigate the effects of carotenoids on the visual performance of mice. Using OptoMotry, a device to measure visual function in rodents, we examined the effect of zeaxanthin, lutein, and β-carotene on visual performance of various BCO knockout mice. We then transgenically expressed the human zeaxanthin-binding protein GSTP1 (hGSTP1) in the rods of *bco2*$^{-/-}$ mice to examine if delivering more zeaxanthin to retina will improve their visual function further. The visual performance of *bco2*$^{-/-}$ mice fed with zeaxanthin or lutein was significantly improved relative to control mice fed with placebo beadlets. β-Carotene had no significant effect in *bco2*$^{-/-}$ mice but modestly improved cone visual function of *bco1*$^{-/-}$ mice. Expression of hGSTP1 in the rods of *bco2*$^{-/-}$mice resulted in a 40% increase of retinal zeaxanthin and further improvement of visual performance. This work demonstrates that these “macular pigment mice” may serve as animal models to study carotenoid function in the retina.

Keywords

Carotenoid; Lutein; zeaxanthin; visual performance; spatial frequency; contrast sensitivity

1. Introduction

Macular carotenoids are yellow xanthophyll pigments that accumulate in the human retina with extremely high concentration in the foveal area [1–3]. These carotenoids have been identified as lutein, zeaxanthin, and *meso*-zeaxanthin [4–7], of which lutein and zeaxanthin originate from the diet, whereas *meso*-zeaxanthin comes mainly from an isomerization reaction of lutein in the retinal pigment epithelium (RPE) [8, 9]. The uptake of macular

*To whom correspondence should be addressed: Paul S. Bernstein, MD, PhD, Moran Eye Center, University of Utah, 65 Mario Capecchi Drive, Salt Lake City, UT 84132, U. S. A., Tel.: 801-581-6078, Fax: 801-581-3357, paul.bernstein@hsc.utah.edu.

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carotenoids has been reported to be a selective and active absorption process involving many transporter proteins and enzymes [1, 10–14]. Glutathione S-transferase Pi isoform (GSTP1) and steroidogenic acute regulatory domain protein 3 (StARD3), are the two carotenoid-binding proteins responsible for the specific retinal distribution of zeaxanthin and lutein, respectively [15, 16]. Many clinical trials and studies have demonstrated that carotenoid supplementation can prevent and reduce the risk of many human eye diseases such as age-related macular degeneration (AMD) [17–19].

It has been well documented that supplementation with lutein and zeaxanthin can improve visual performance of both normal subjects and patients with eye diseases [20–26]. Loughman et al. reported that significant improvements in visual acuity were found at the sixth month in normal subjects fed with a mixture of lutein, zeaxanthin, and *meso*-zeaxanthin [27]. A randomized, double-blind, placebo-controlled, 1-year interventional study in 120 Chinese drivers demonstrated that lutein supplementation can increase contrast sensitivity and decrease glare disability [28]. It was also reported that visual acuity of cataract patients supplemented with lutein improved about one line on the Snellen visual acuity chart in comparison with a placebo group [29]. Stringham and Hammond found that light scattering was greatly reduced for short wavelength monochromatic light in subjects with high levels of macular carotenoids, suggesting that macular carotenoids can mitigate glare disability [20]. The visual benefits of macular carotenoids are attributed to their optical properties, antioxidant effects, and other biological mechanisms [30, 31]. Macular carotenoids are thought to be able to reduce chromatic aberration, light scatter, and glare disability by absorbing blue light [1, 32]. They also can quench free radicals and maintain retinal health [13, 33]. Until now, however, it has been difficult to test hypothesis to examine the mechanisms underlying improvement of visual performance by carotenoid supplementation due to the lack of small mammal models capable of reproducibly accumulating substantial levels of carotenoids in their retinas [34–37]. In 2014, our group discovered that zeaxanthin can be deposited in the retina of mice deficient in the β-carotene oxygenase 2 (BCO2) enzyme, generating so-called “macular pigment mice” [38]. More recently, we were also able to deliver comparable amounts of lutein and lower levels of β-carotene to the retinas of these *bco2*$^{-/-}$ mice, while *bco1*$^{-/-}$ mice were superior for delivery of β-carotene to the retina [39]. These results have been confirmed by other research groups, and no morphological difference is detected between the retinas of wild-type mice and *bco2*$^{-/-}$ mice [40–42]. All these researchers have shown that “macular pigment mice” are likely to be good laboratory animal models to study the effects of carotenoids on visual performance.

In this manuscript, we investigate the effects of zeaxanthin, lutein, and β-carotene on the spatial frequency and contrast sensitivity of rod and cone cells of the “macular pigment mice” using OptoMotry, a device to examine visual function of small animals. Furthermore, we tested if delivering more carotenoids to the retina of transgenic mice expressing the human zeaxanthin binding protein GSTP1 (hGSTP1) in their rod cells will induce further improvement of their visual function.

2. Material and methods

2.1 Animal husbandry and generation.

Bco2$^{-/-}$ and *bco1*$^{-/-}$ mice were bred at the University of Utah vivarium using founders from Case Western Reserve University. To express zeaxanthin-binding protein GSTP1 specifically in the mouse retina, we generated human *GSTP1* transgenic (*hGSTP1-tg*) mice. In brief, an XhoI site was inserted immediately upstream of the translation initiation codon and a ClaI site immediately downstream of the translation stop codon of the cDNA of human *GSTP1* by PCR. The XhoI/ClaI fragment was subcloned into corresponding sites of pRho 4.4 vector to place the human GSTP1 gene under the control of the mouse opsin promoter. In order to track expression of the transgene, a hemagglutinin (HA) tag was placed contiguous with the human GSTP1 cDNA sequence. After direct DNA sequencing, the 5.6-kb trangene construct containing the rhodopsin promoter, human GSTP1 cDNA, a HA-tag, and a mouse protamine polyadenylation signal was isolated from the plasmid by digestion with KpnI and XbaI, then injected into C57BL/6X129 embryos. The embryos were implanted into pseudopregnant foster female mice. Founder mice with transgene integration were identified by PCR, and mated to wildtype C57BL/6 mice to produce mice used for analysis. Subsequently, the *hGSTP1-tg* mice were bred with the *bco2*$^{-/-}$ mice to generate *hGSTP1-tg /bco2*$^{-/-}$ mice with the expectation that they would accumulate more zeaxanthin in their retinas relative to *bco2*$^{-/-}$ mice. All the procedures were approved by appropriate institutional animal care and use committees and were carried out according to National Institutes of Health guidelines.

2.2 Carotenoid-feeding experiments.

Bco2$^{-/-}$, *bco1*$^{-/-}$, *hGSTP1-tg* , and *hGSTP1-tg /bco2*$^{-/-}$ mice were employed in the carotenoid-feeding experiments in which *bco2*$^{-/-}$ mice were treated with lutein, zeaxanthin, or β-carotene, *bco1*$^{-/-}$ mice were treated with β-carotene, and *hGSTP1-tg* and *hGSTP1-tg / bco2*$^{-/-}$ mice were treated with zeaxanthin. In each experiment, 3-month-old mice were divided into two groups and fed with carotenoid beadlet chow (~2.6 mg per mouse per day; DSM, Kaiseraugst, Switzerland) or placebo beadlet chow for 4 weeks after first receiving a vitamin A-deficient chow (AIN-93, (TestDiet, Richmond, IN)) for 4 weeks to help promote carotenoid uptake. Then, their visual performance and carotenoid contents were examined.

2.3 Carotenoid extraction and analysis by HPLC.

Carotenoids in liver and serum, as well as ocular tissues of the mice were extracted and analyzed as before [39]. Briefly, the ocular tissue and liver samples were extracted three times with tetrahydrofuran containing 0.1% butylated hydroxytoluene by sonication at 5°C to 10°C for 30 minutes each time. Combined extracts were evaporated to dryness under vacuum at room temperature. To extract carotenoids from serum, ethanol containing 0.1% butylated hydroxytoluene was added into the samples to precipitate the proteins, and then ethyl acetate was added to extract the carotenoids. The sample was centrifuged at 2,000 x g for 5 minutes at 4°C, and the supernatant phase was collected. Then the sample was extracted with ethyl acetate two more times and extracted with hexane once. The collected supernatants were combined and dried down under vacuum. Finally, the dried residue was re-dissolved in HPLC mobile phase and centrifuged at 2000 x g for 10 minutes, and the supernatant was injected into the HPLC system. HPLC separations were performed on a

silica-based nitrile bonded column (25 cm length × 4.6 mm internal diameter; 5-μm spherical particle (Regis Chemical, Morton Grove, IL)). The eluent consisted of an isocratic mixture of hexanes (75%), dichloromethane (25%), methanol (0.3%), and N, N-diisopropylethylamine (0.1%). The column flow rate was 1 mL/min. The column temperature was maintained at 25°C, and the monitoring wavelength was 445 nm.

2.4 OptoMotry.

3- to 4-month-old mice (n=7 to 15/ group) were employed to test spatial visual acuity using the OptoMotry system (Cerebral Mechanics, Lethbridg, AB, Canada). Briefly, individual mice were placed on a platform centered in a quad-square formed by four inward facing computer screens, and their movements were monitored by an overhead video camera. Photopic measurements were conducted under illuminance of around 165 lux. Scotopic measurements were carried out in infrared light with the LCD displays masked with 5 layers of ND16 Lee299 filters. During the detection of spatial frequency threshold, the rotation speed and contrast were kept at 12 degrees/s, and 100%, respectively, while the frequency was kept at 0.19 cycle/degree in the examination of contrast sensitivity. All experiments had concurrent control mice fed with placebo chow.

2.5 RT-PCR.

Total RNA was prepared from mouse retinas. cDNA was synthesized using SuperScriptIII reverse transcriptase. PCR to detect the expression of the human GSTP1 transgene was performed with 1μl RT reaction as template. Primers were as follows: forward, 5′-TGG TGG ACA TGG TGA ATG ACG G −3′; and reverse, 5′- AGC GTA GTC TGG GAC GTC GTA TG −3′ to yield a 393 bp fragment.

2.6 Western blots and Immunohistochemistry.

Protein samples were separated on 4−15% gradient SDS−PAGE and transferred to 0.45 μm nitrocellulose membranes. After blocking with 5% nonfat dried milk, the membranes were incubated with primary and secondary antibodies. The dilution ratios were 1:1000 and 1:2000, respectively. The membranes were developed using ECL Plus Western blot detection reagents (GE Healthcare Bio-Sciences, Pittsburgh, PA). In the immunohistochemistry experiments, sections of perfusion-fixed monkey eyes were processed as described [43] with the addition of heating sections in a solution of 10 mM sodium citrate, pH 6, at 95°C (5 min) prior to blocking with 10% normal donkey serum in PBS-T. Antibodies used were: Anti-GSTP1 and anti-HA-tag antibodies from Alpha Diagnostic International (San Antonio, TX); anti-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

2.7 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) and Stata/15.1 statistical software (StataCorp, College Station, TX, USA).

3. Results

To test the effects of carotenoids on visual performance of mice, *bco2*$^{-/-}$ mice were fed with zeaxanthin, lutein, or β-carotene for one month, and then their photopic and scotopic spatial frequencies and contrast sensitivities were quantified using OptoMotry. Of note, the photopic and scotopic parameters represent visual function of the cone and rod systems, respectively. Higher values of spatial frequency correspond to better visual acuity, whereas smaller contrast sensitivities are indicative of better visual function. Figure 1 shows that zeaxanthin supplementation significantly increased the spatial frequency and contrast sensitivity of both rod and cone systems in the *bco2*$^{-/-}$ mice. In comparison with mice of the placebo group, the rod and cone spatial frequencies are increased around 15% in mice fed with zeaxanthin, while the rod and cone contrast sensitivities improved around 20% and35%, respectively. Like zeaxanthin supplementation, lutein supplementation can significantly increase the visual performance of *bco2*$^{-/-}$ mice, except for their scotopic contrast sensitivity (Figure 2). The extent of improvement by lutein supplementation is similar to zeaxanthin in regard to the rod and cone spatial frequency and rod contrast sensitivity. Lutein also was able to significantly improve the cone contrast sensitivity but was slightly less than zeaxanthin, around 20%.

β-Carotene is not a human macular carotenoid, and only a trace amount of β-carotene is detected in the human retina overall. However, we still investigated the effects of β-carotene on visual performance as it is the precursor of retinal, a key molecule involved in vision, and it also shares the blue light filtering property of lutein and zeaxanthin. Figure 3 demonstrates that no significant improvement in visual function was detected in *bco2*$^{-/-}$ mice fed with β-carotene relative to the control mice. This is not surprising because BCO1, the critical cleavage enzyme for β-carotene, is still present in the *bco2*$^{-/-}$ mice. Our previous study revealed that the content of β-carotene is only around 5 to 10% of that of lutein and zeaxanthin in the retina of *bco2*$^{-/-}$ mice, while the content of β-carotene in the retinas of *bco1*$^{-/-}$ mice supplemented with β-carotene is comparable to the contents of lutein or zeaxanthin in the retina of supplemented *bco2*$^{-/-}$ mice [39]. Therefore, we further investigated if β-carotene can improve the visual performance of *bco1*$^{-/-}$ mice. Figure 4 shows that β-carotene supplementation can significantly improve the spatial frequency and contrast sensitivity of the cone system but not the rod system. There were a 4% and 9% improvements detected in the photopic spatial frequency and contrast sensitivity of mice fed with β-carotene relative to the control mice. All these OptoMotry data indicate that, of these three carotenoids, zeaxanthin is the best at improving the visual performance of mice, especially for cone contrast sensitivity.

Next, we tested if delivering more zeaxanthin to the retina of mice will further improve their visual performance. In order to deliver more zeaxanthin to the retina of mice, we transgenically expressed the human zeaxanthin-binding protein GSTP1 (hGSTP1) in the retina of *bco2*$^{-/-}$ mice by crossing an *hGSTP1* transgenic mouse line (*hGSTP1-tg*) with the *bco2*$^{-/-}$ mice. Figure 5 shows the transgene construct and the expression of hGSTP1. cDNA encoding hGSTP1 protein was placed under the control of the mouse rhodopsin promoter, which drives hGSTP1 protein expression specifically in rods. Confocal immunolocalization of the expressed HA-tag showed robust expression of human GSTP1 throughout rods, from

the outer plexiform layers (OPL) to outer segments (OS). The *hGSTP1-tg* mice were mated to *bco2*$^{-/-}$ mice in order to generate *hGSTP1-tg/bco2*$^{-/-}$ mice.

We then performed a zeaxanthin-feeding experiment, in which ~ 3-month-old *hGSTP1-tg/bco2*$^{-/-}$ and *bco2*$^{-/-}$ mice were fed with DSM-beadlet diets for one month. Figure 6 shows the carotenoid contents detected by HPLC in this feeding experiment. Zeaxanthin content was ~0.84 ng/ pair of retinas in the *hGSTP1-tg /bco2*$^{-/-}$ mice, which is around 40% higher than that of *bco2*$^{-/-}$mice. Meanwhile, there was no significant difference was found between the zeaxanthin contents of RPE/choroids, serum, or livers of *hGSTP1-tg /bco2*$^{-/-}$ mice and those of *bco2*$^{-/-}$ mice. In addition, we could not deliver zeaxanthin into the retina of the *hGSTP1-tg* mice. This is because the carotenoid cleavage enzyme BCO2 is still functional in these mice, so zeaxanthin molecules will be broken down before arrival at the retina.

We next examined the impact of zeaxanthin on the visual performance of *hGSTP1-tg / bco2*$^{-/-}$ mice. 3-month-old *hGSTP1-tg /bco2*$^{-/-}$ mice were divided into two groups and fed with or without zeaxanthin for 4 weeks. We then examined their visual performance using OptoMotry (Figure 7). Comparing with the control mice, the rod and cone spatial frequency and contrast sensitivity were significantly improved in the *hGSTP1-tg /bco2*$^{-/-}$ mice fed with zeaxanthin, and similar improvements in the rod and cone spatial frequency were seen when comparing the *hGSTP1-tg / bco2*$^{-/-}$ and the *bco2*$^{-/-}$ mice. An obvious improvement was found in the rod contrast sensitivity of *hGSTP1-tg /bco2*$^{-/-}$ mice in contrast to the *bco2*$^{-/-}$ mice. This increase in *hGSTP1-tg /bco2*$^{-/-}$ mice is about 35% while it is only 20% in the *bco2*$^{-/-}$ mice. This may be ascribed to the contribution of the zeaxanthin-binding protein GSTP1 expressed specifically in the rod cells. It is also shows that zeaxanthin supplementation caused a 45% increase in the cone contrast of *hGSTP1-tg /bco2*$^{-/-}$, which is about 1.3times as high as the *bco2*$^{-/-}$ mice. No significant difference was found between the visual performance of *hGSTP1-tg/bco2*$^{-/-}$ control mice and *bco2*$^{-/-}$ control mice.

4. Discussion

Besides protection against light-induced oxidative damage in the retina, improving visual performance is another primary function of the macular carotenoids. It is well known that carotenoid supplementation can improve the visual performance of both normal subjects and those with eye disease, but there is always concern that these are subjective psychophysical tests that could be influenced by subject and examiner bias. Our previous studies have established that transgenic “macular pigment mice” whose carotenoid cleavage enzymes have been selectively knocked out can serve as animal models for bioavailability and bio-efficacy of retinal carotenoids. In the present work, we demonstrate that supplementation with lutein and zeaxanthin improves the spatial frequency and contrast sensitivity of mice, especially the contrast sensitivity, mimicking the results of the recent clinical trials in humans [44, 45]. This validates that *bco2*$^{-/-}$ mice can be used to investigate the functional benefits of the macular carotenoids.

Our investigations revealed several new insights into the effects of carotenoids on visual function. We found that xanthophyll carotenoids can significantly improve the visual performance of both rod and cone cells, while β-carotene just slightly enhances the visual

performance of cone cells in mice (Figures 1–4). Supplementation with lutein and zeaxanthin dramatically increased the contrast sensitivity of cone cells of *bco2*$^{-/-}$ mice, and zeaxanthin was around 1.2±0.19 time stronger than lutein. (Figures 1–2). We also examined β-carotene's effects on visual performance in *bco2*$^{-/-}$ mice, and no improvement was detected which we ascribed to the low retinal content of β-carotene in these mice. Our previously published study has shown that only trace amounts of retinal β-carotene can be detected in the *bco2*$^{-/-}$ mice because β-carotene's main cleavage enzyme, BCO1, is still active [39]. To raise β-carotene to a comparable level of lutein and zeaxanthin in the retina, we fed β-carotene to mice deficient in the BCO1 enzyme, and the OptoMotry data show that β-carotene can slightly increase the visual function of cone cells.

GSTP1 and StARD3 have been identified to be the zeaxanthin-binding protein and lutein-binding proteins in the human retina, respectively. In this work, we also took advantage of this property of GSTP1 and examined if delivering more zeaxanthin to the retina of mice will further improve their visual performance. The human GSTP1 protein was transgenically expressed in the retina of *bco2*$^{-/-}$ mice, causing the retinal carotenoid content to increase around 40% more than the *bco2*$^{-/-}$ mice under the same feeding conditions (Figures 5–6). Since this specific expression is driven by the mouse rhodopsin promoter, the human GSTP1 proteins have been robustly expressed in the rod cells, and correspondingly, more zeaxanthin should be deposited there. This may be responsible for the dramatic improvement in the contrast sensitivity of rod cells in *hGSTP1-tg/bco2*$^{-/-}$ mice after zeaxanthin supplementation (Figure 7), supporting the idea that increasing the retinal carotenoid levels can improve visual function. Of course, to study the role of macular carotenoids in visual performance further, we should selectively elevate the carotenoid level of various cone cells because, after all, the majority of the macular carotenoids are present in an area dominated by cone cells. We also found that no carotenoid was accumulated in the retina of *hGSTP1-tg* mice, indicating that BCO2 is a critical enzyme for the presence of carotenoid in the retina. All of these results are consistent with our previous findings that the relative inactivity of the human BCO2 enzyme is responsible for the accumulation of xanthophyll carotenoids in the human macula and that the high cleavage activity of mouse BCO2 is responsible for the failure of wild-type mice to accumulate any retinal carotenoids even after extreme systemic doses and overexpression of carotenoid-binding proteins in the retina.

Of all of the possible mechanisms for carotenoid improvement of visual performance in humans and "macular pigment mice" (light filtering, antioxidant, and other neural and biochemical mechanisms), blue-light filtering by these yellow pigments is the most straightforward. Our OptoMotry results showed a rank order of visual performance of *hGSTP1-tg/bco2*$^{-/-}$ (zeaxanthin-fed) > *bco2*$^{-/-}$ (zeaxanthin-fed) ≈ *bco2*$^{-/-}$ (lutein-fed) > *bco1*$^{-/-}$ (β-carotene-fed) > *bco2*$^{-/-}$ (β-carotene-fed) >> any mouse (placebo-fed), yet retinal content of the intact carotenoids in our transgenic mice was *hGSTP1-tg/bco2*$^{-/-}$ (zeaxanthin-fed) > *bco2*$^{-/-}$ (zeaxanthin-fed) ≈ *bco2*$^{-/-}$ (lutein-fed) ≈ *bco1*$^{-/-}$ (β-carotene-fed) > *bco2*$^{-/-}$ (β-carotene-fed) >> any mouse (placebo-fed) (Figure 8). This disconnect between performance and carotenoid content can be explained by the fact that transgenic BCO knockout mice generate considerable amounts of yellow oxidative products when fed lutein or zeaxanthin which can also be deposited in the mouse retina, while β-carotene-fed BCO knockout mice do not generate these yellow metabolites [39]. As can be seen in Figure 8, if

we sum the intact carotenoids with their yellow metabolites, the rank order of total carotenoids aligns with the visual performance results rankings. In addition, in a separate control experiment using older mice, we found that the cone visual function of *bco2*$^{-/-}$ mice was decreased 20% to 30% compared to WT (*bco2*$^{+/+}$) mice of the same age. From the visual function data of *bco2*$^{-/-}$ mice fed with zeaxanthin (Figure 1), we can see that the visual function of *bco2*$^{-/-}$ mice fed with zeaxanthin was improved 20% to 35% relative to the control mice on placebo diet, suggesting that carotenoid supplementation may improve the impaired visual function of *bco2*$^{-/-}$ mice.

Our results in transgenic mice are consistent with the effect of macular carotenoids on visual performance revealed in recent human clinical trials and studies. This implies that these transgenic “macular pigment mice” may be successfully employed to further dissect the molecular mechanisms underlying the beneficial effects of the macular carotenoids on visual function.

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Abbreviations

AMD	Age-related macular degeneration
BCO1	β-carotene oxygenase 1
BCO2	β-carotene oxygenase 2
GSTP1	Glutathione S-transferase Pi isoform
HA	Hemagglutinin
HPLC	High-performance liquid chromatography
RPE	Retinal pigment epithelium
StARD3	Steroidogenic acute regulatory domain protein 3

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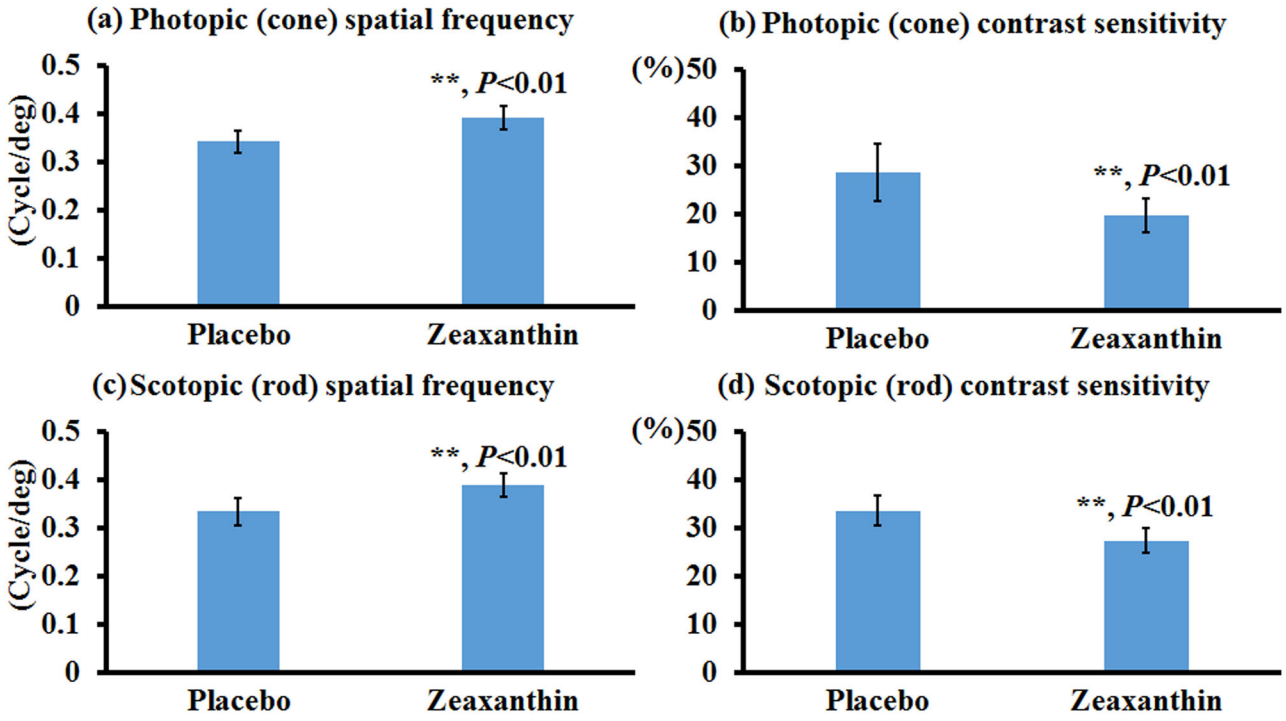

Figure 1. Visual performance measured by OptoMotry in *bco2*$^{-/-}$ mice with and without zeaxanthin supplementation.

Zeaxanthin supplementation significantly improves the visual function of *bco2*$^{-/-}$ mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 10 mice were used in each group. **, $P<0.01$.

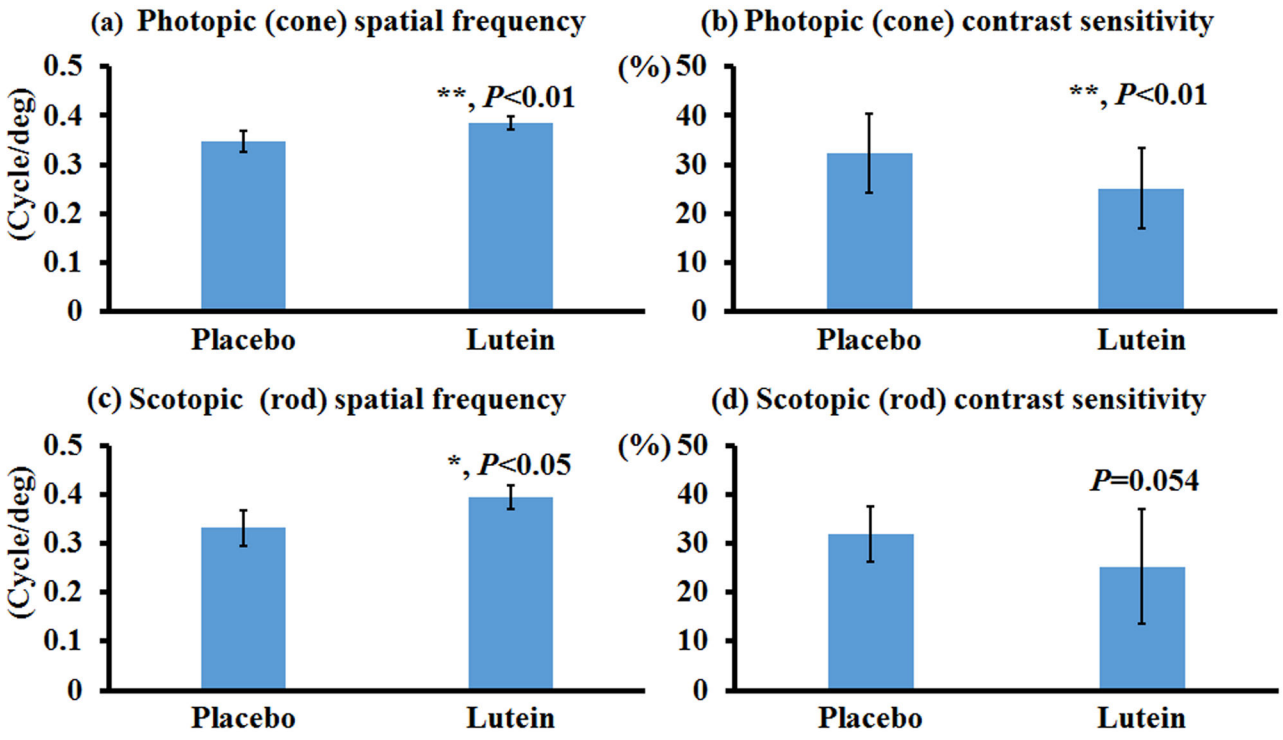

Figure 2. Visual performance measured by OptoMotry in *bco2*$^{-/-}$ mice with and without lutein supplementation.

Lutein supplementation significantly improves *bco2*$^{-/-}$ mice's visual function except for the contrast sensitivity of the rod cells. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 15 mice were used in each group. *, $P<0.05$; **, $P<0.01$.

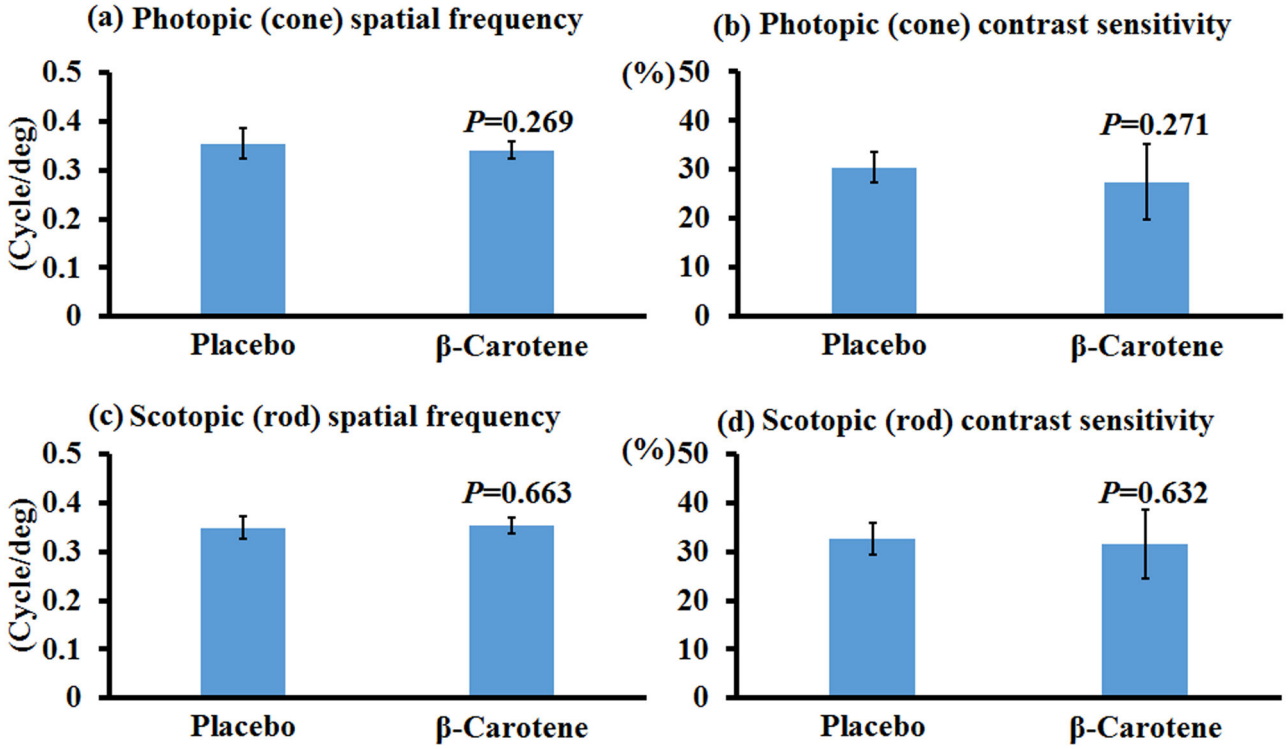

Figure 3. Visual performance measured by OptoMotry in *bco2*$^{-/-}$ mice with and without β-carotene supplementation.

β-Carotene supplementation has no significant effect on the visual performance of *bco2*$^{-/-}$ mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 10 mice were used in each group. *, $P<0.05$.

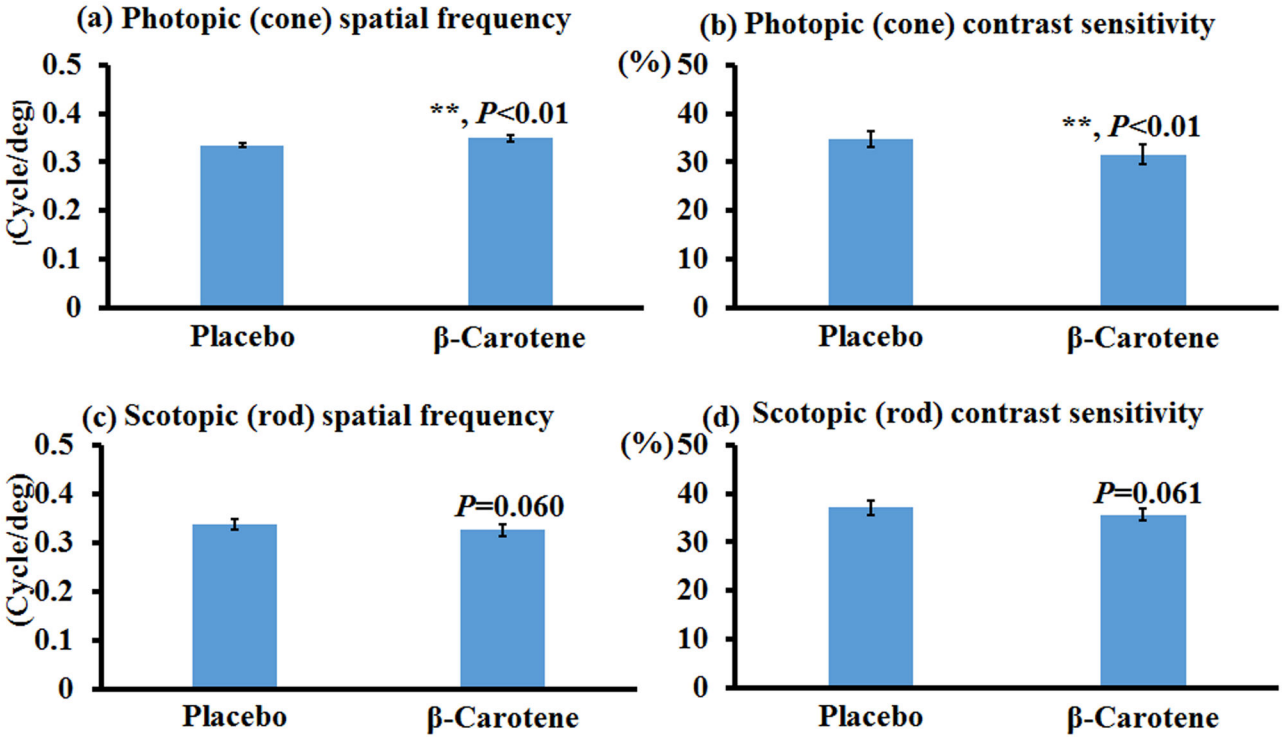

Figure 4. Visual performance measured by OptoMotry in *bco1*$^{-/-}$ mice with and without β-carotene supplementation.

β-Carotene supplementation slightly improves the visual performance of *bco1*$^{-/-}$ mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 7 and 8 mice were used in β-carotene and placebo groups, respectively. *, $P<0.05$.

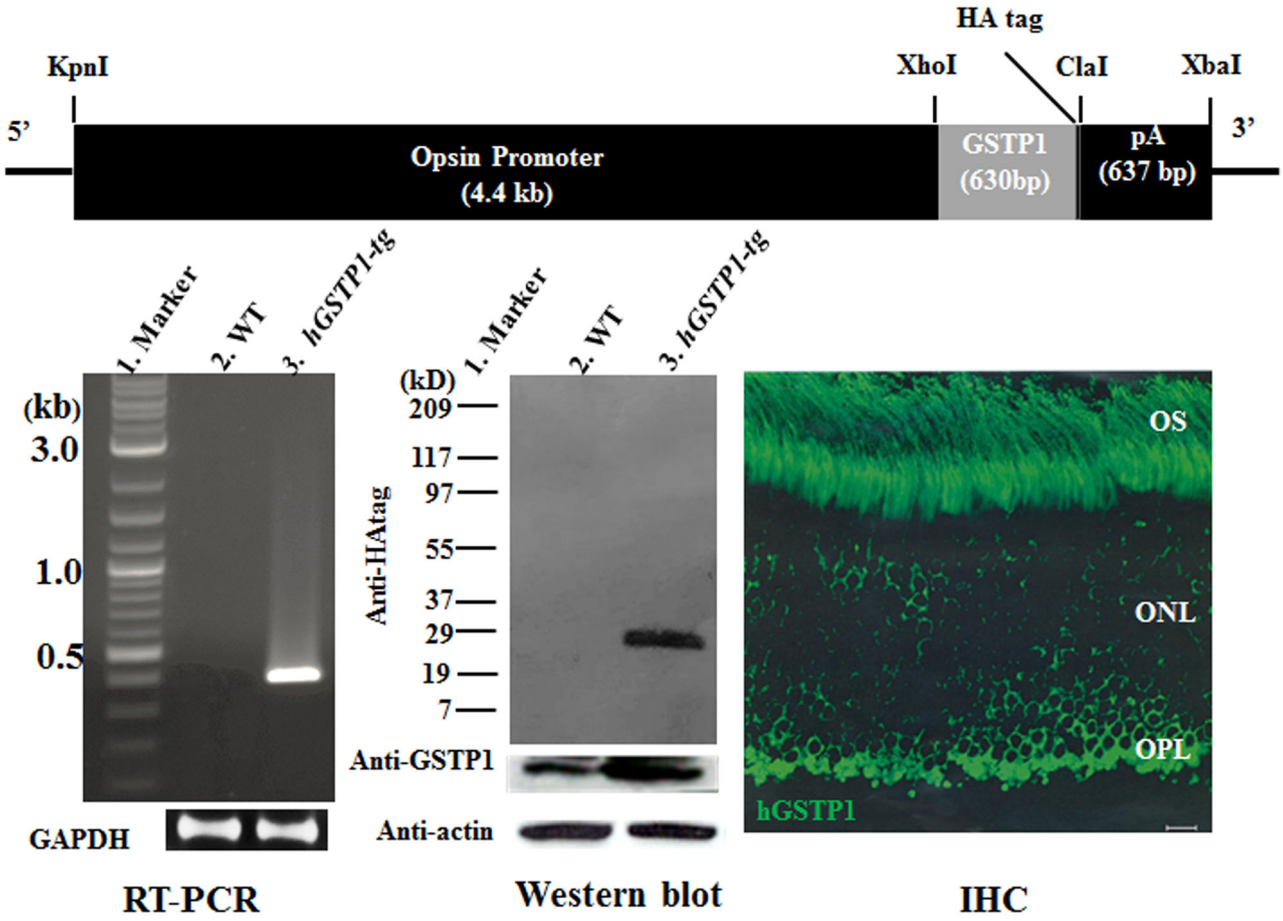

Figure 5. Generation of transgenic (*hGSTP1-tg*) mice expressing the human zeaxanthin-binding protein GSTP1 specifically in the retina.

The transgene construct (upper panel). RT-PCR reveals presence of human *GSTP1* in the transgenic mouse retina. Lane 1. Amplicon size marker; 2. Wildtype C57BL/6 mice (WT); 3. *hGSTP1-tg* mice. Samples are normalized by GAPDH (lower left panel). Immunoblot results of antibody directed against the HA-tag versus total protein extract from pooled mouse retinas. Lane 1. Protein size marker; 2. C57BL/6 mice (WT); 3. *hGSTP1-tg* mice. Samples are normalized by actin (lower middle panel). Immunolocalization with antibody to HA-tag (green) in a 1-month-old *hGSTP1-tg* mouse retina (far right panel). OS, outer segments; ONL, outer nuclear layers; OPL, outer plexiform layers.

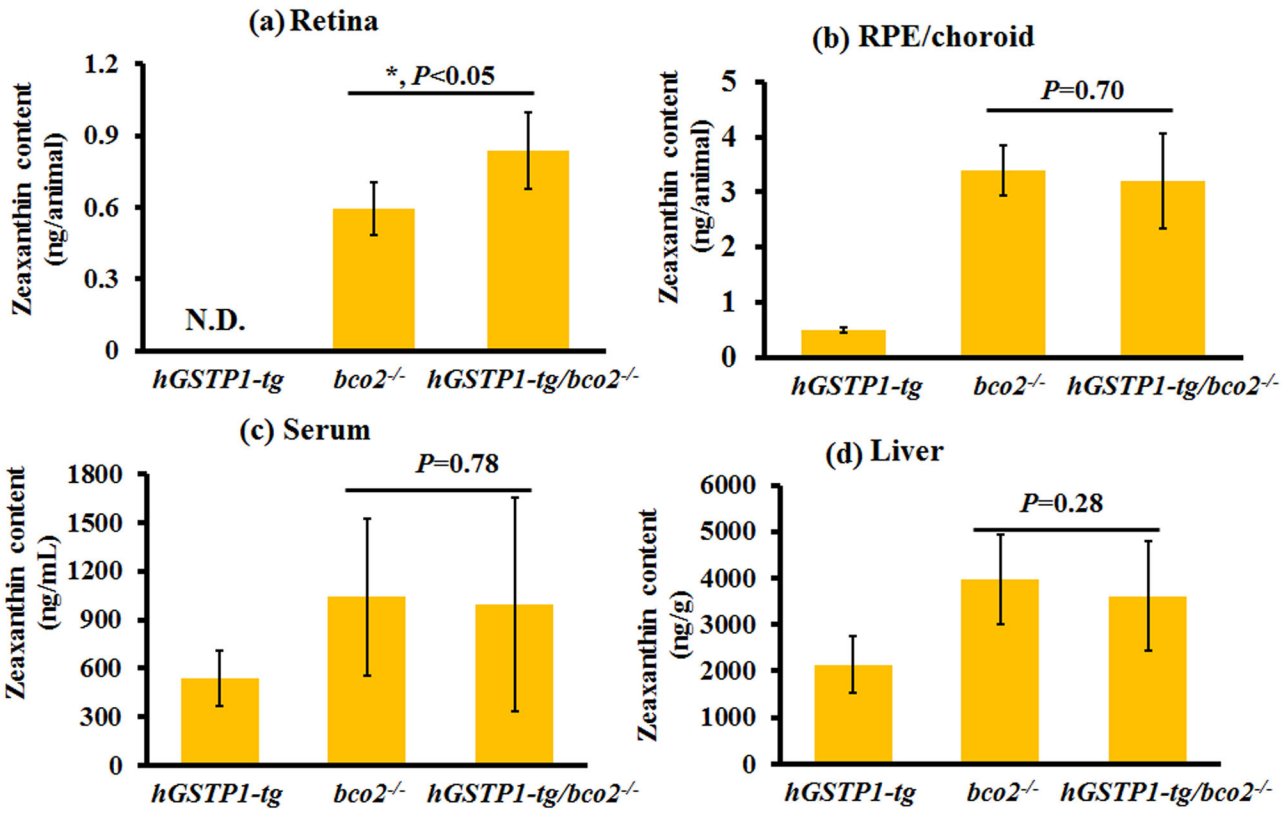

Figure 6. Contents of zeaxanthin detected in the tissues of *hGSTP1-tg*, *bco2*$^{-/-}$ and *hGSTP1-tg/bco2*$^{-/-}$ mice.

The expression of zeaxanthin-binding protein GSTP1 specifically in the retina of *bco2*$^{-/-}$ mice significantly increased the retinal carotenoid contents. 8 to 10-week-old mice (n=25/genotype) were kept on DSM zeaxanthin beadlet chow (1 g zeaxanthin/kg chow) 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, N.D., not detectable. *, $P<0.05$; **, $P<0.01$.

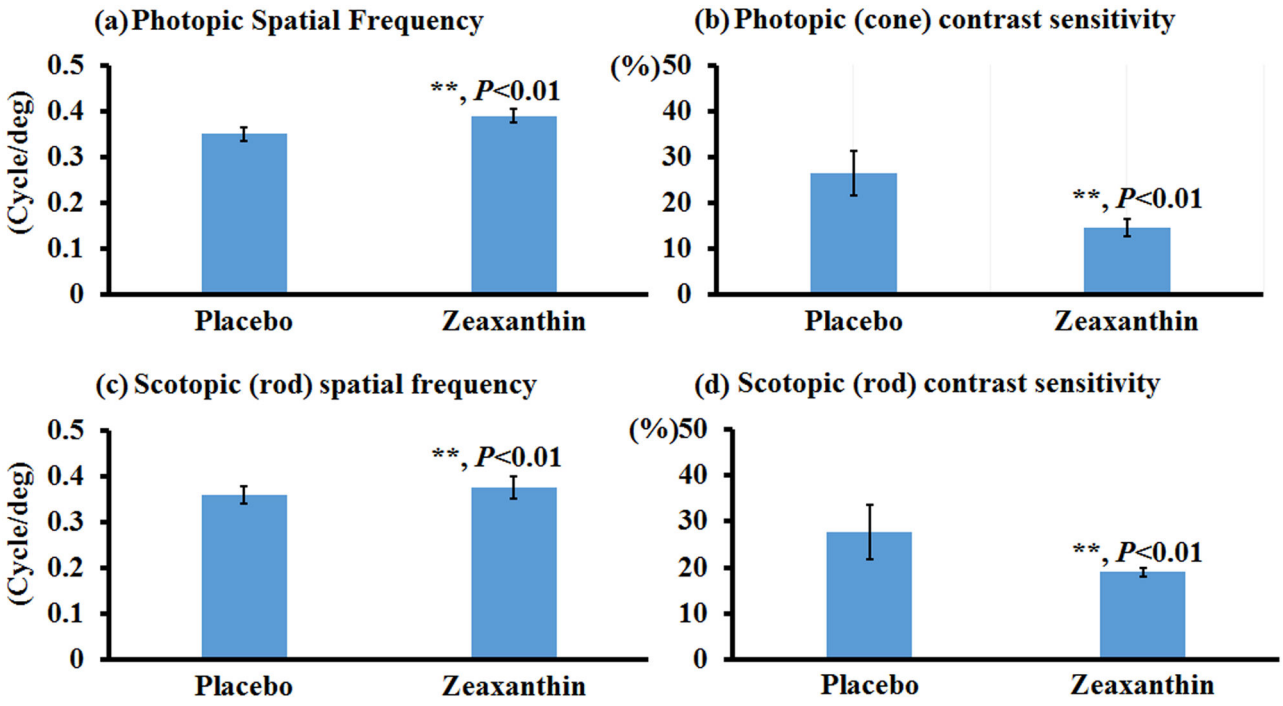

Figure 7. Visual performance measured by OptoMotry in *hGSTP1-tg/bco2*$^{-/-}$ mice with or without zeaxanthin supplementation.

Zeaxanthin supplementation significantly improves the visual function of *hGSTP1-tg/bco2*$^{-/-}$ mice, especially the rod contrast sensitivity. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 11 and 14 mice were used in the zeaxanthin and placebo groups, respectively. **, P<0.01.

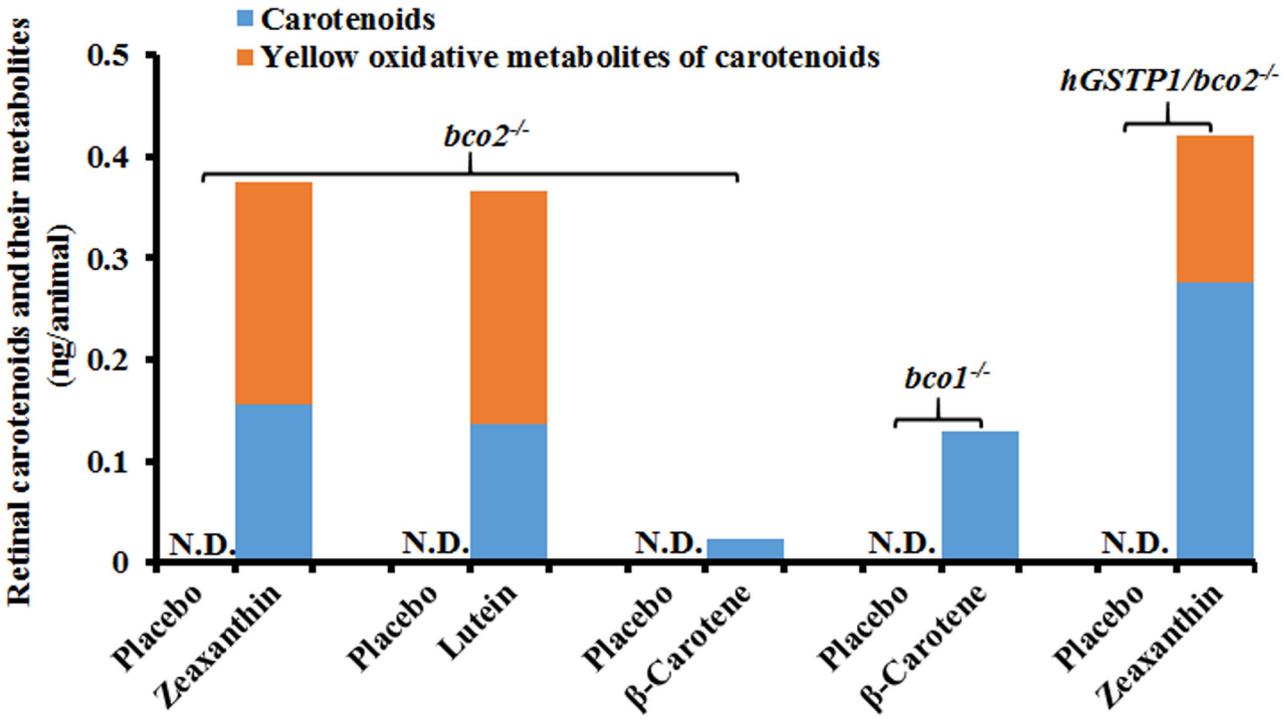

Figure 8. Contents of carotenoids and their yellow oxidative metabolites in the retinas of the mice used in the visual performance experiments.

The yellow oxidative metabolites of carotenoids were detected in the mice fed with zeaxanthin or lutein but not β-carotene, and their amounts were estimated using authentic standard of lutein or zeaxanthin as these metabolite compounds have not been identified yet. The number of mice in each feeding group varies from 7 to 15, and the retinas from 3 to 7 animals were pooled together for carotenoid analysis. N.D., not detectable.