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SUPPLEMENTATION WITH MACULAR CAROTENOIDS IMPROVES VISUAL PERFORMANCE
OF TRANSGENIC MICE

by

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ABSTRACT

Previous work has shown that carotenoid supplementation can improve visual acuity in humans. However, there is yet to be a viable rodent model to verify the effects of carotenoids on the visual performance of laboratory animals. The Bernstein Lab at the Moran Eye Center recently demonstrated that mice deficient in β -carotene oxygenase 2 (BCO2) and/or β -carotene oxygenase 1 (BCO1) enzymes are able to accumulate carotenoids in their retinas. This allows an investigation into the effects of carotenoids on visual function of mice. Using OptoMotry, a device used to measure visual function in rodents, we examined the effect of zeaxanthin, lutein, and β -carotene on visual performance of various BCO knockout mice. To further investigate the potential positive effects of supplementation, we transgenically expressed the human zeaxanthin-binding protein GSTP1 (hGSTP1) in the rods of *bco2*^{-/-} mice to investigate if accumulating more zeaxanthin in the retina would improve visual performance further. In summary, the visual function of *bco2*^{-/-} mice supplemented with lutein or zeaxanthin improved significantly over control mice fed with placebo chow. β -Carotene has no significant effect on the visual performance of *bco2*^{-/-} mice, but slightly improved visual function of *bco1*^{-/-} mice. Additionally, with expression of the hGSTP1 in the rods of *bco2*^{-/-} mice, 40% more zeaxanthin was found in the retina which resulted in even greater improvements of visual acuity. The presented research verifies that “macular pigment

mice” are a viable laboratory model to study the effects of carotenoids on visual systems.

TABLE OF CONTENTS

ABSTRACT	ii
INTRODUCTION	4
METHODS	8
RESULTS	11
DISCUSSION	19
REFERENCES	26
PUBLISHED WORK	30 (22)

INTRODUCTION

Carotenoids are one of the major groups of phytochemicals, molecules which are produced by plants. Carotenoids are characterized as carotenes if they consist only of hydrogen and carbon. If they are oxidized to any degree, they are referred to as xanthophylls. Three carotenoids used in this paper are β -carotene, and xanthophyll carotenoids lutein and zeaxanthin. These molecules consist of large, conjugated polyene chain backbones with a beta-ionone ring at each end. These conjugated backbones allow the carotenoid molecule to be very efficient antioxidants, diffusing the energy in toxic reactive oxygen species (ROS) and absorbing harmful blue light (Bernstein et al., 2016). ROS are highly reactive radicals and formal charge bearing oxygens that can react with a long list of biomolecules. For example, if any protein undergoes a chemical reaction, it has a large probability of changing the shape, and therefore changing or losing the function of that protein. Additionally, these ROS can alter DNA structures leading to higher likelihoods of cancers and other mutations. In summary, having unregulated reactions in a cell can be detrimental to the health of that cell.

Interestingly, carotenoids are found in nearly all organisms in varying locations and varying concentrations, following the idea that having an effective radical-scavenging molecule is evolutionarily advantageous (Maoka, 2011). Humans are rather unique in their utilization of carotenoids in that they distribute them in high concentrations to the *macula lutea*, creating the yellow spot that is centered at the fovea (Bone et al., 1992; Handelman et al., 1992). The carotenoid species that are used in the macula of humans are lutein, zeaxanthin, and *meso*-zeaxanthin, and are referred to as macular pigments (MP). Lutein and zeaxanthin are obtained

by diet, and *meso*-zeaxanthin is an isomerization of zeaxanthin. The optimal wavelength that MP absorb light is at 455 nm, with a range between 400-550 nm (Bernstein et al., 2016). This is interesting because blue and violet light (390-495 nm) have been found to create ROS (Godley et al., 2005). Carotenoids act both as an effective mechanism of quenching these high energy ROS, and absorbing the light that would make more of these species.

As investigative research focuses more and more on MP, an increasingly large consensus suggests that MP are crucial for high visual acuity due to its blue light-filtering properties, reduction in chromatic aberration, veiling luminance, and blue haze. (Hammond et al., 2014; Loughman et al., 2012). Additionally, MP may protect against AMD because of these optical characteristics in connection with the antioxidant capacity of the three carotenoids (Sabour-Pickett et al., 2012). In an almost elegant manner, the human eye has selected for distribution of a particular molecule that prevents and extinguishes ROS. Not only do they reduce oxidative damage and help prevent ROS from being created in the first place, MP also increase visual acuity.

In most animals, carotenoids are distributed in many tissues, including liver, serum, adipose tissue, and brain (Bernstein et al., 2016). Distribution of carotenoids to the ocular tissues is a relatively rare occurrence. This phenomenon is only observed in avian and primate species (Bernstein et. al, 2016). However, carotenoids are a rather common biomolecule in nature. The reason that so few species have retinal carotenoids is still under debate. Furthermore, one theory revolves around the story of carotenoid cleavage enzymes and carotenoid-binding proteins. When carotenoids are consumed, most are cleaved by BCO1 and/or BCO2, carotenoid

oxygenase enzymes (Bernstein et al., 2016). These cleavage enzymes break down the carotenoids, resulting in several products used throughout the body. Of the 700 different variants of carotenoids known to exist, only 15-30 make it into the human blood stream, and only two of these make it to the human retina (Bernstein et al., 2016). There, the carotenoids are taken up and accumulated in the retina of the eye by GSTP1 and STARD3 proteins (Bhosale et al., 2004; Li et al., 2011). Interestingly, humans have a very inactive form of BCO2 enzyme in the retina. The binding affinity between carotenoid and human retinal BCO2 is around 10-40 times weaker than the same enzyme in other species, causing human retinal BCO2 to be an inactive carotenoid cleavage enzyme (Li et al., 2014). We have previously confirmed that mice deficient in the BCO2 gene can accumulate lutein and zeaxanthin in ocular tissues. Though this paints a very clear picture as it relates to humans, the pathway of ocular carotenoid accumulation for avian and other species is not well understood.

The Age-Related Eye Disease Study 2 (AREDS 2) conducted by the National Eye Institute (NEI) in 2006 has concluded that supplementation of lutein and zeaxanthin has been found to reduce the risk of Age-Related Macular Degeneration (AMD). As this is the second study by the NEI, carotenoids have been accepted as molecules that can positively affect visual acuity and longevity of vision. Related studies would be very important to conduct. However, the experimental models that accurately represent human MP are limited.

Primates are excellent models for these kinds of studies. Their MP is very similar to humans, and the mechanism for distribution of these molecules is also comparable. However, there are a large amount of difficulties that can arise from using primates as clinical models.

We have recently found that mice lacking the BCO2 gene can accumulate carotenoids in the retina. Mice have been used as research models for centuries due to their strikingly similar anatomy to humans and ease of care, among many other desirable traits. In this thesis, 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.

In the following work, I will convey the extent to which I participated in this project, add my own commentary, and reflect on the results and possible future work that can be done regarding this subject matter. As an undergraduate, I have worked in a lab at the Moran Eye Center of the University of Utah for the past two years. The Principal Investigator for this work is Dr. Paul Bernstein, M.D., Ph.D. I work on a daily basis with Dr. Binxing Li, who is a postdoctoral fellow in Bernstein’s Lab. At the time that I joined the lab, Drs. Bernstein and Li had successfully created and verified “macular pigment mice” as a viable model to test the effects of carotenoids on visual systems. The next step, as explained above, was to determine the effects of such vitamins on visual performance.

Over the course of roughly 18 months, Dr. Li, myself, and nine others worked together to verify our hypothesis that supplementation with carotenoids do in fact improve visual performance in macular pigment mice. The results of said work has recently been published in the Journal of

Archives of Biochemistry and Biophysics. I am included as second author of the work, as well as a UROP recipient for the Spring 2019 semester to continue research on this same subject matter. As stated, the purpose of this thesis is to explore my involvement, participation, and reflections of this project.

Attached is a copy of the published manuscript. The original work will be referred to extensively.

METHODS

Much of the preliminary work regarding the husbandry and feeding of the mice was done by Dr. Li and my colleague Fu-Yen Chang. Fu-Yen specializes in genetics and worked closely with Dr. Li in the tasks of animal husbandry and Genotyping. It was necessary for me to not be involved with the feeding of the mice as to keep me blind to which groups of mice were fed the carotenoid chow. The OptoMotry System (Cerebral Mechanics, Lethbridg, AB, Canada) was used to determine the visual acuity and contrast sensitivity of the mice. Each group had 7-15 mice being 3-4 months old. Placebo chow was given to another group of mice to act as a control for each test group. Please refer to the published work under bullet number 2.4 *OptoMotry* for greater detail of these tasks.

I must admit that I did not enjoy doing this portion of the experiment. The test required 10-15 minutes of testing for each mouse. However, the beginning stages of my learning required 30-60 minutes per mouse. There were around 130 individual mice to be tested in both scotopic and photopic conditions. Only with a great amount of practice and patience was a 10-15 minute

test possible. As one may suspect, many experiments had to be repeated due to my inconsistency at the beginning of the experiment. As I learned, I was able to extract excellent data, but it was only after many, many hours of practice. One observation that I had during these tests was that the grounds for determining if the mouse was able to detect changes were subject to the observer. I highly suspect that if someone who was also proficient at using this system tested the same mice that I did, that we would obtain different results. However, the beauty of this system is that it allows for *comparison* of two groups. The hypothesis predicted an increase in visual acuity, not a specific level of sight being obtained. This differs with the Snell Eye Test which can produce consistent levels of sight among humans. Though as investigators we would like to be able to determine a consistent level of acuity that is consistent between observers, a comparison of difference is adequate to show visual performance increases.

After OptoMotry experiments were conducted, the mice needed to be sacrificed and dissected. Though rather gruesome, this portion was the most enjoyable. As a team of four, three of my colleagues and I took posts each with a specific task. We collected blood, liver, brain, and eye samples from each mouse. An aspect of this work that was in contrast to the acquisition of visual acuity and contrast sensitivity of the mice was human interaction. I found very quickly that I much preferred to work with people rather than mice.

After the collection of tissues was complete, we needed to extract the carotenoids from the various tissues and determine their relative levels. For each, a homogenization process had to be conducted. For liver and brain samples, that involved weighing certain amount of tissue

from each sample and using a tissue homogenizer to liquefy them one by one. This took an extensive amount of time including several late nights homogenizing. After tissues were homogenized, an extraction protocol was followed to separate the carotenoids from the various samples. A detailed outline of this protocol can be found in the published work under bullet number *2.4 Carotenoid extraction and analysis by HPLC*. I personally did the majority of the homogenizations and many of the extractions.

The process of analyzing the retina and retinal pigment epithelium (RPE) tissues of the mice required an additional step. Each eye sample had to be dissected and the RPE and retina needed to be separated. I personally did this for every mouse sample in the experiment. This was my favorite part of the work that we did. I really enjoyed the challenge of dissecting such a small eye, and it seemed very similar to surgery which is something that I am very interested in. I also have been involved with flat mounting other mouse eye samples for use in other experiments.

Another aspect of the research that I was in charge of was protein analysis by Western Blot and Immunohistochemistry. Since my start at the lab I have been the person who runs these experiments in the lab.

I also assisted Dr. Li in the analysis process after the data was collected. We collaborated to synthesize the data into a coherent result.

RESULTS

To verify that carotenoid supplementation can have an effect on the visual function of *bco2*^{-/-} mice, groups were fed β -carotene, lutein, or zeaxanthin for one month. The OptoMotry device was used to test the changes in visual function of the various groups. Two parameters were used to test the cone and rod systems separately, namely photopic and scotopic conditions, as well as spatial frequency and contrast sensitivity separately. Higher visual acuity corresponded with higher spatial frequency scores and lower contrast scores corresponded with better visual acuity with regards to contrast sensitivity.

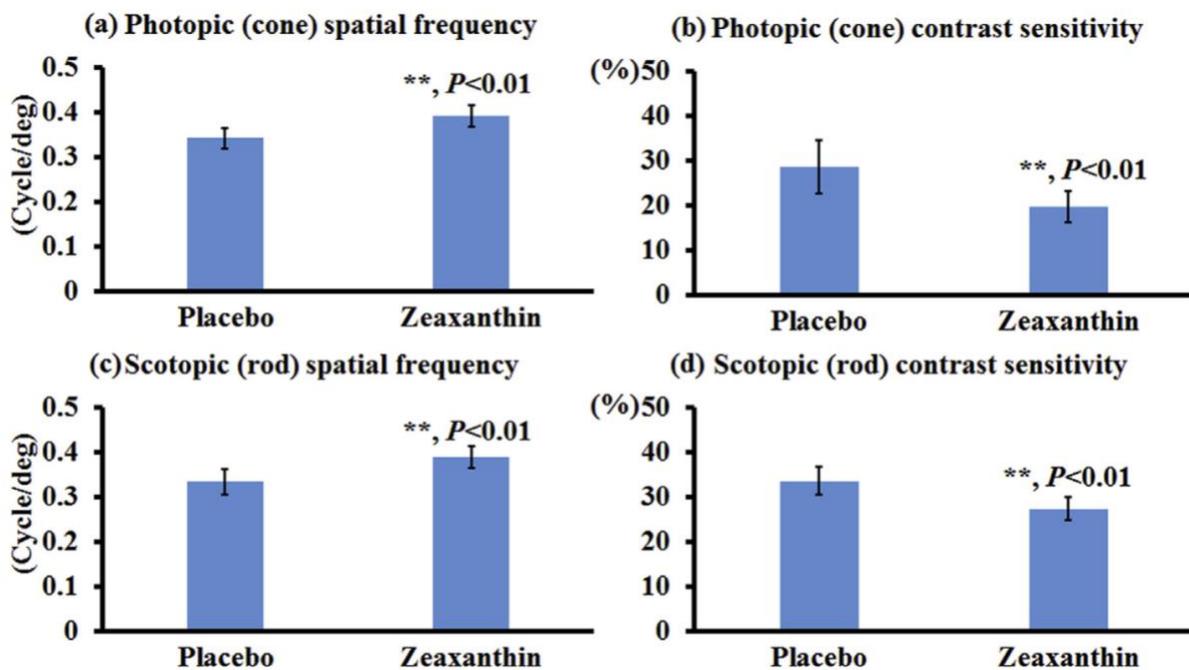


Fig. 1 Visual performance measured by OptoMotry in *bco2*^{-/-} mice with and without zeaxanthin supplementation. Zeaxanthin supplementation significantly improves the visual function of *bco2*^{-/-} mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means \pm SD; 10 mice were used in each group. **, $P < 0.01$.

Fig. 1 shown above demonstrates that zeaxanthin supplementation significantly increases spatial frequency and contrast sensitivity in both photopic and scotopic conditions of *bco2*^{-/-} mice. When compared to the placebo fed *bco2*^{-/-} mice, spatial frequency sensitivity increased by about 15% in both cone and rod pathways, and contrast sensitivities in cone and rod systems increased between 20% and 35% respectively.

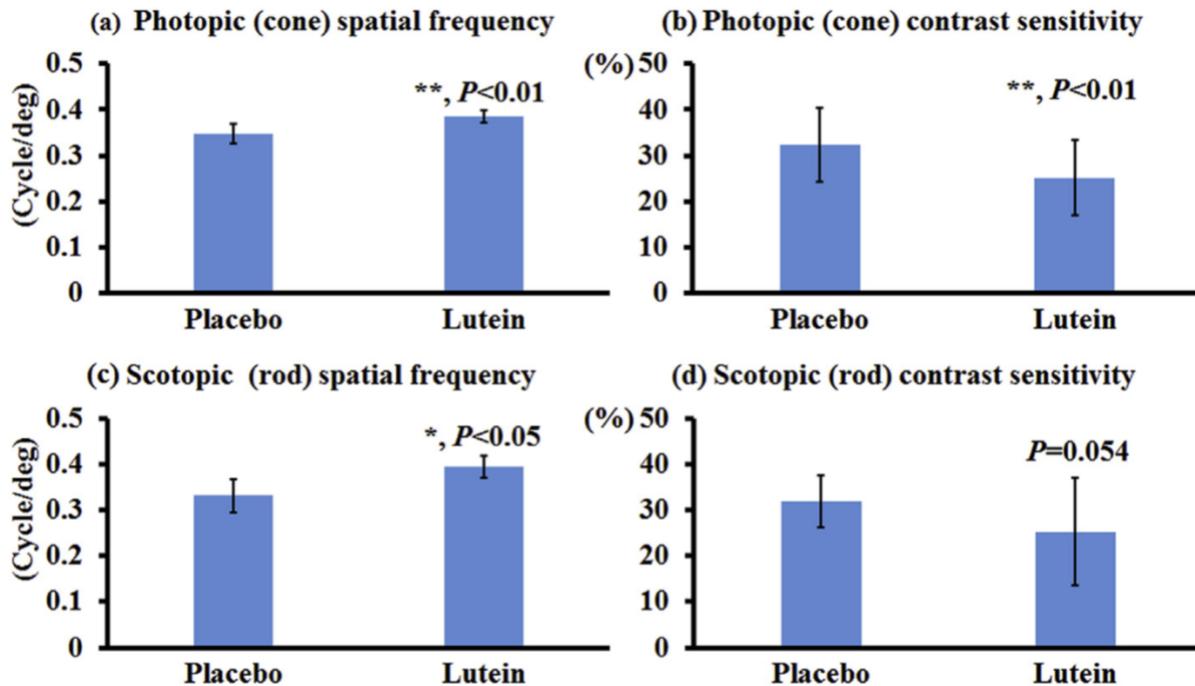


Fig. 2. Visual performance measured by OptoMotry in *bco2*^{-/-} mice with and without lutein supplementation. Lutein supplementation significantly improves *bco2*^{-/-} mice's visual function except for the contrast sensitivity of the rod cells. (a) Photopic spatial frequency; (b) Photopic contrast 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.

Additionally, lutein also significantly increased visual acuity in both spatial and contrast sensitivity in cone systems, but not in contrast sensitivity in rod systems (Fig. 2). The differences between the carotenoid supplemented group and the placebo group is similar to that of

zeaxanthin, but only improved cone contrast sensitivity by 20%, slightly less than the impact of zeaxanthin.

With respect to the large error bars associated with the contrast sensitivity, especially in scotopic conditions, I believe the problem with the data is not due to the carotenoid not having effect on the mice. I would estimate that much of the lack of agreement among the data has to do with the fact that two observer's results of the mice were averaged. However, as I discussed before, the OptoMotry system is most useful for comparison and not consistent values across observers. Therefore, the difference between the placebo and variable groups of mice may be similar, but the actual acuity and contrast scores associated with each group can vary between observers. I would then say that if I were able to retest the lutein fed *bco2*^{-/-} mice for contrast sensitivity, there is a high likelihood that I would be able to make dependable observations. As you can deduce, the line was barely crossed to label the scotopic contrast tests as insignificant. Though the fact that the OptoMotry system has difficulty reproducing actual values between observers does not deem the system any less useful. Being able to measure the difference between two groups is sufficient to conclude that a certain therapy does in fact improve function.

β -Carotene is found only in trace amounts in the human retina, and therefore has very little effect on the overall health and visual capacity of our visual system. However, β -carotene is the precursor to retinal, a very important molecule to vision, and it also shares the light-absorbing character of lutein and zeaxanthin.

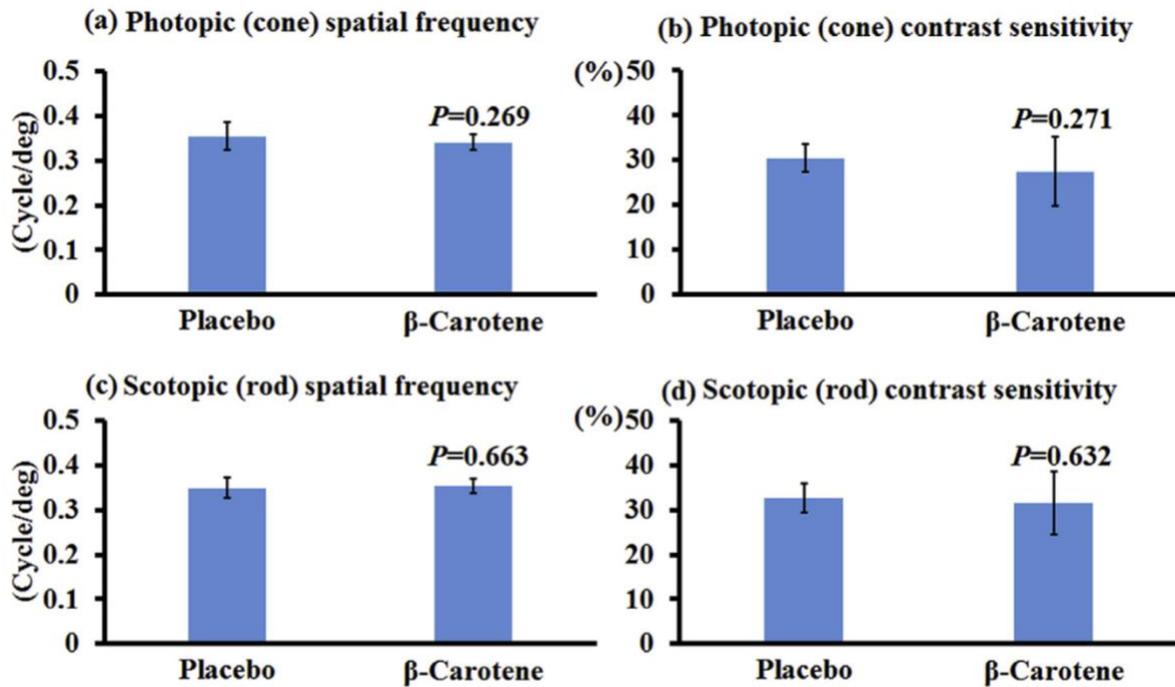


Fig. 3. Visual performance measured by OptoMotry in *bco2*^{-/-} mice with and without β -carotene supplementation. β -Carotene supplementation has no significant effect on the visual performance of *bco2*^{-/-} mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means \pm SD; 10 mice were used in each group. *, $P < 0.05$.

Fig. 3 shows that β -carotene has no significant effect on the visual performance of *bco2*^{-/-} mice. A large part of this is due to the fact that BCO1, the main cleavage enzyme that reacts with β -carotene, is still active in *bco2*^{-/-} mice. In previous work done by our lab, it has been shown that β -carotene is distributed to the eye in ratios of 10-15% of the levels of lutein and zeaxanthin in the retina of *bco2*^{-/-} mice. Furthermore, the levels of β -carotene in the retinas of *bco1*^{-/-} mice is comparable to that of lutein and zeaxanthin in the retinas of *bco2*^{-/-} mice (Li et al., 2017).

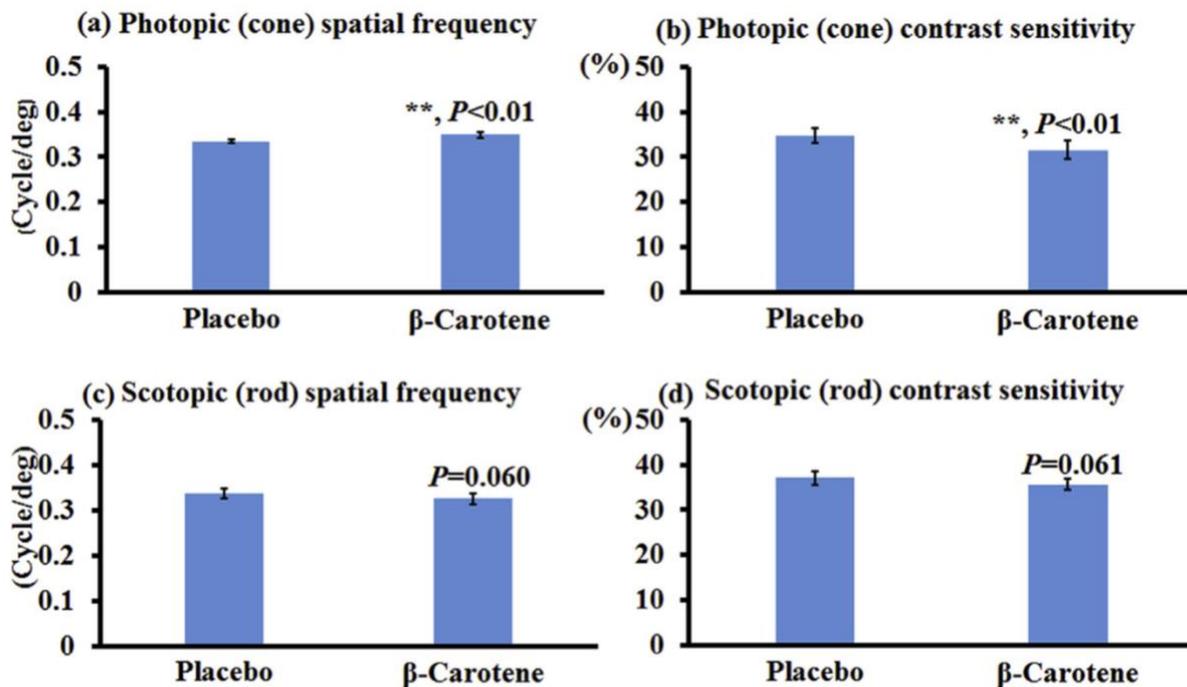


Fig. 4. Visual performance measured by OptoMotry in *bco1*^{-/-} mice with and without β - carotene supplementation. β - Carotene supple- mentation slightly improves the visual performance of *bco1*^{-/-} mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means \pm SD; 7 and 8 mice were used in β - carotene and placebo groups, respectively. *, P < 0.05.

Fig. 4 shows that β -carotene supplementation can significantly increase the visual performance in cone systems of *bco1*^{-/-} mice. However, the increase of ability over the placebo fed mice is very small, being 4% and 9% with respect to spatial and contrast sensitivity. Given an overarching view of the data, zeaxanthin improves visual performance the most, with lutein giving similar results, and β -carotene only having small improvements compared to placebo fed mice.

Considering that zeaxanthin is the most effective of the three carotenoids in improving vision, we wanted to investigate the possibility of further increasing visual performance by distributing

more zeaxanthin to the retina. To do so 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.

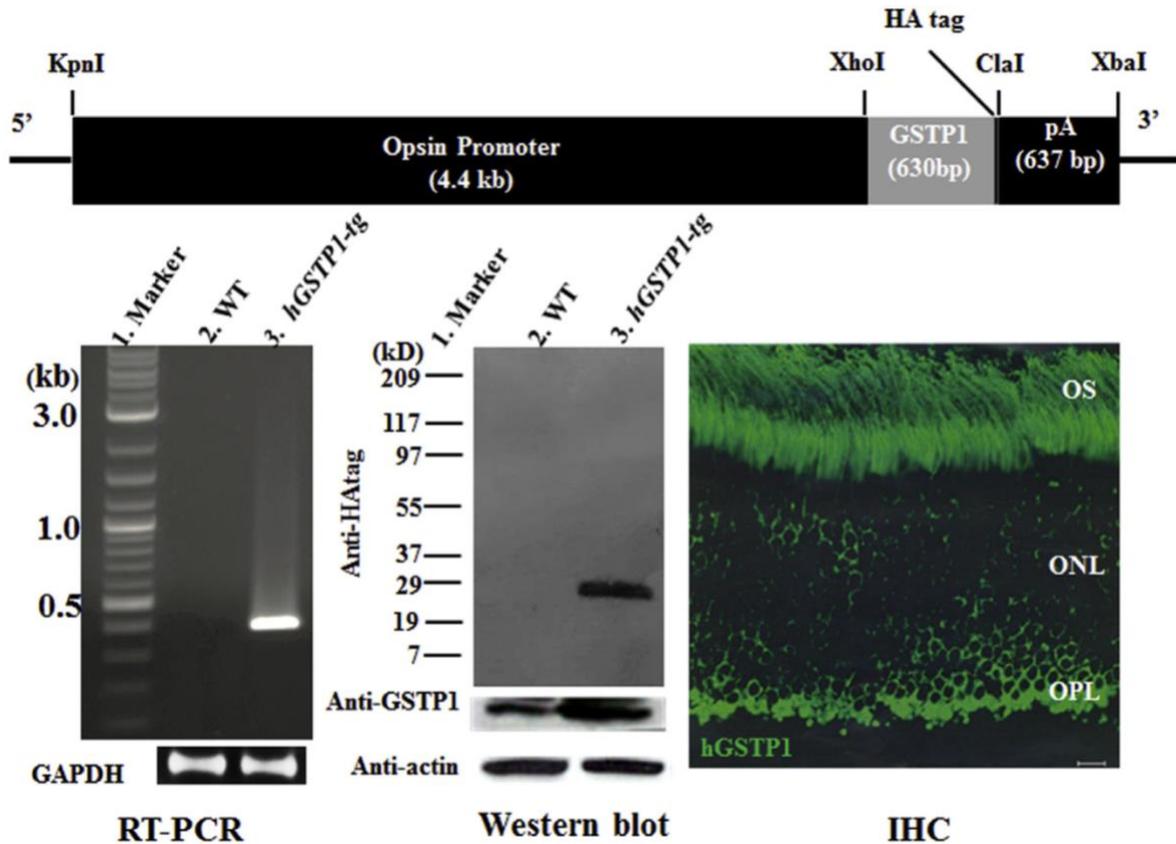


Fig. 5. Generation of transgenic (*hGSTP1-tg*) mice expressing the human zeaxanthin-binding protein GSTP1 specifically in the retina. The transgene construct (upper panel). RT-PCR reveals presence of human GSTP1 in the transgenic mouse retina. Lane 1. Amplicon size marker; 2. Wildtype C57BL/6 mice (WT); 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.

Fig. 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 the rod cells, 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.

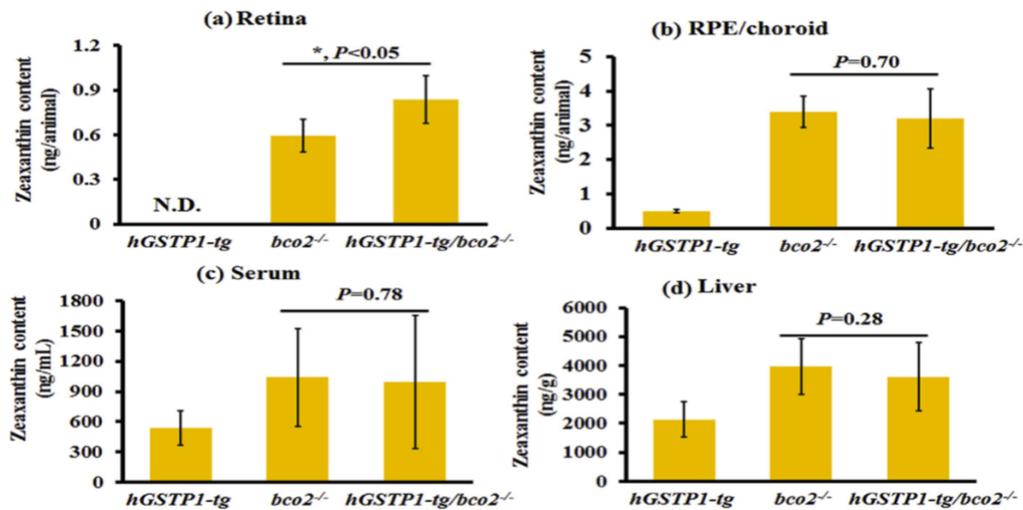


Fig. 6. Contents of zeaxanthin detected in the tissues of *hGSTP1-tg*, *bco2^{-/-}* and *hGSTP1-tg/bco2^{-/-}* mice. The expression of zeaxanthin-binding protein GSTP1 specifically in the retina of *bco2^{-/-}* mice significantly increased the retinal carotenoid contents. 8 to 10-week-old mice (n = 25/genotype) 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.

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. Fig. 6 shows the levels of zeaxanthin in several tissues of *hGSTP1-tg*, *hGSTP1-tg/bco2^{-/-}* and *bco2^{-/-}* mice. We determined these levels by HPLC. The difference found between the three different mice is significant. As predicted, *hGSTP1-tg* mice will not distribute zeaxanthin to the retina since the

BCO2 cleavage enzyme is still active. Additionally, *bco2*^{-/-} mice were found to have appreciable amounts of zeaxanthin in the retinal tissue and *hGSTP1-tg/bco2*^{-/-} mice had significantly more zeaxanthin, roughly 40% more than *bco2*^{-/-} mice. There was no difference between zeaxanthin levels found in the RPE/choroid, serum, and liver of these mice.

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.

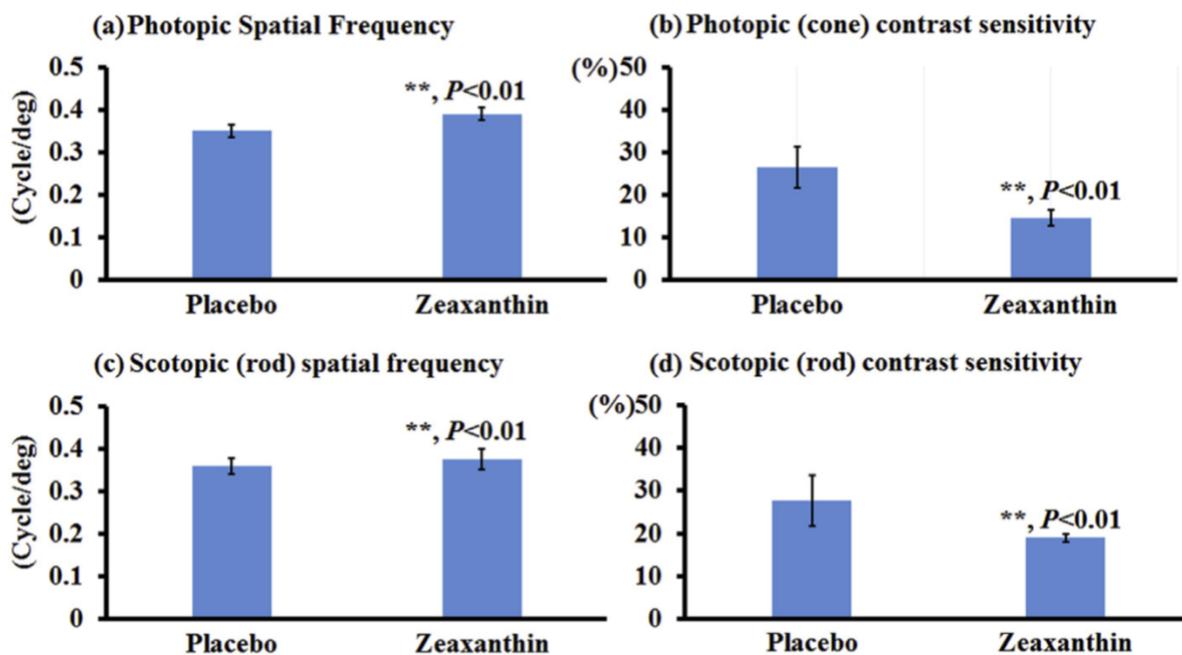


Fig. 7. Visual performance measured by OptoMotry in *hGSTP1-tg/bco2*^{-/-} mice with or without zeaxanthin supplementation. Zeaxanthin supplementation significantly improves the visual function of *hGSTP1-tg/bco2*^{-/-} mice, especially the rod contrast sensitivity. (a) Photopic spatial frequency; (b) 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.

Fig. 7 shows the results of the OptoMotry experiments. As shown, the variable group showed very significant improvements over the placebo group. The improvements with the *hGSTP1-tg/bco2^{-/-}* mice fed with zeaxanthin are similar to those found in the *bco2^{-/-}* mice fed with zeaxanthin. There is an apparent difference found in the contrast sensitivity of *hGSTP1-tg/bco2^{-/-}* and *bco2^{-/-}* mice in scotopic conditions, suggesting that the former further improves rod system acuity when compared to the latter. The difference between is significant where *hGSTP1-tg/bco2^{-/-}* mice improved vision by 35%, while the *bco2^{-/-}* mice only increased by 20%. This may be attributed to the zeaxanthin binding protein GSTP1 being expressed uniquely in the rod cells. The data also demonstrates that the *hGSTP1-tg/bco2^{-/-}* mice had an increase of 45% in photopic contrast sensitivity, this being about 1.3 times greater than the *bco2^{-/-}* mice. There was no difference found between the visual acuity of placebo *hGSTP1-tg/bco2^{-/-}* mice and placebo *bco2^{-/-}* mice.

DISCUSSION

Carotenoids are known to be effective scavengers of reactive oxygen species. The importance of such a mechanism is apparent. However, the visual performance enhancing role that carotenoids play in ocular systems is also a primary function of these vitamins. It has been established previously that carotenoid supplementation can improve visual performance in humans (Akuffo et al., 2017; Stringham et al., 2017). Until this point a viable non-primate model for testing the effects carotenoid supplementation has on visual performance has not been found. Previous studies in the Bernstein lab have demonstrated that so-called “macular pigment mice” are effective models for distributing ocular carotenoids at appreciable levels (Li et al., 2014). In this investigation, we establish that carotenoid supplementation does in fact

improve visual acuity and contrast sensitivity in *bco2*^{-/-} mice. This is especially clear in the area of contrast sensitivity. These results verify the viability of *bco2*^{-/-} mice being used as experimental models to further research the benefits of carotenoid supplementation.

Additionally, our research has shed greater light on the visual enhancing properties of ocular carotenoids. We have shown that xanthophyll carotenoids, namely lutein and zeaxanthin, can dramatically increase spatial and contrast sensitivity when distributed in appreciable amounts to the ocular tissues of mice, with zeaxanthin being the most effective of the two (Figs. 1-2). Furthermore, we have also demonstrated that β -carotene only slightly increases visual acuity when distributed to ocular tissues. β -Carotene was also shown to not accumulate in the retinas of *bco2*^{-/-} mice. This can be attributed to the fact that BCO1, the cleavage enzyme responsible for degrading many carotenoids before reaching ocular tissues, is still active in these mice. This is further demonstrated by *bco1*^{-/-} mice supplemented with β -carotene does accumulate the molecule in the eye (Figs. 1-4).

Carotenoids are often cleaved in the digestive tract before reaching the eye, but the distribution of these vitamins to the eye can be facilitated by other binding proteins in the eye. GSTP1 and StARD3 have been identified to be the zeaxanthin-binding protein and lutein-binding proteins in the human retina, respectively. In order to further demonstrate the visual enhancing properties of zeaxanthin, we used this model to further improve the vision of mice. By expressing the human GSTP1 in *bco2*^{-/-} mice, we were able to extend the amount of zeaxanthin found in the eye when fed the same amount of carotenoid. The measure of which is substantial, increasing concentrations by 40% compared to non-GSTP1 bearing mice (Figs. 5-6).

This may be the reason why *hGSTP1-tg/bco2^{-/-}* mice see significantly better than *bco2^{-/-}* mice (Fig. 7). This observation also supports the proposition that increase zeaxanthin concentrations in ocular tissues can increase visual performance. Among discoveries regarding the human GSTP1 protein is the fact that *hGSTP1-tg* mice do not accumulate carotenoids to the retina, suggesting that BCO2 is the critical cleavage enzyme in this pathway. Taking this into consideration, it supports previous claims from our lab that BCO2 activity is responsible for preventing wild type mice from accumulating ocular carotenoids. Additionally, considering that the human BCO2 protein is 10-40 times less efficient than the mouse form, it would follow the idea that humans distribute carotenoids to the macula because the critical cleavage enzyme has very low activity levels comparatively.

With regards to the OptoMotry results, the following is a list of which groups performed the best with respect to each other, listing them from greatest to least improvement:

- 1- *hGSTP1-tg/bco2^{-/-}* (zeaxanthin-fed)
- 2- *bco2^{-/-}* (zeaxanthin-fed)
- 3- *bco2^{-/-}* (lutein-fed)
- 4- *bco1^{-/-}* (β -carotene-fed)
- 5- *bco2^{-/-}* (β -carotene-fed)
- 6- Any placebo mouse (significantly lower; placebo-fed)

Additionally, retinal content of carotenoids are as follows, again ranking from greatest to least amount:

- 1- *hGSTP1-tg/bco2^{-/-}* (zeaxanthin-fed)
- 2- *bco2^{-/-}* (zeaxanthin-fed) \approx *bco2^{-/-}* (lutein-fed) \approx *bco1^{-/-}* (β -carotene-fed)

3- *bco2*^{-/-} (β -carotene-fed)

4- Any placebo mouse (significantly lower; placebo-fed)

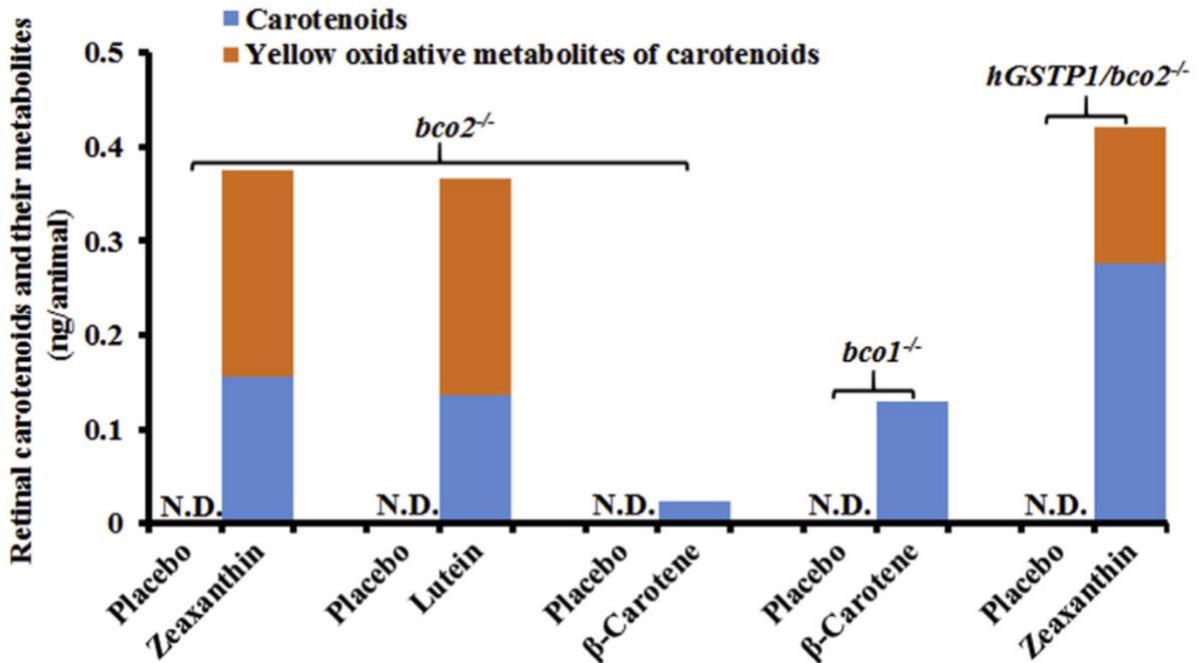


Fig. 8. Contents of carotenoids and their yellow oxidative metabolites in the retinas of the mice used in the visual performance experiments. The yellow oxidative metabolites of carotenoids were detected in the mice fed with zeaxanthin or lutein, but not β -carotene, and their amounts were estimated 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.

Interestingly, there is an inconsistency with regard to carotenoid content in the retina and visual performance, seeing that β -carotene significantly underperformed compared to zeaxanthin and lutein. This is explained by BCO knockout mice generating 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 (Li et al., 2017). To then correct the inconsistency, if we sum the carotenoid and yellow pigment content in the retinas we find that this then reflects the order of visual performance (Fig. 8).

Our investigations have produced results similar to those found in recent studies completed with human test subjects: carotenoid supplementation can significantly improve visual performance. Furthermore, we can confidently suggest the use of the so called “macular pigment mice” as a viable model to further investigate the effects and possibilities of carotenoids on visual systems.

Future investigations regarding this new finding are exciting. One such would be to investigate if a dual treatment of lutein and zeaxanthin would further increase the positive effects observed on visual acuity. Additionally, we have investigated and demonstrated that expression of human GSTP1 can improve zeaxanthin accumulation at the retina and therefore improve vision, but we have not yet tested the lutein binding protein StARD3 at this time. A similar experiment of BCO knockout mice also expressing the human StARD3 gene with lutein supplementation would be interesting.

This work has demonstrated the rather drastic improvements that carotenoids can have on vision. However, this is not the only use of carotenoids in the eye. As discussed, these vitamins are used as effective ROS scavengers. I hypothesize that these can act as a defense mechanism against cellular degradation, perhaps conserving visual function at a higher level for a longer amount of time. This can be seen as a preventative treatment to vision loss in general due to aging of the eye, but also due to degeneration of the macula. The National Health Institute found a correlation between carotenoid supplementation and lower morbidity of AMD. Though AMD is proliferative among humanity, little is known regarding causation of the disease. I

would propose that high levels of damage, among which would logically be damage due to ROS, proceed AMD and can be reduced by supplementation with carotenoids. As introduced, up until this point there has not been a viable investigative model in which carotenoid supplementation experiments could be conducted. As we have demonstrated, the “macular pigment mouse” can now fill this role. In so doing, we are now able to investigate if carotenoids can indeed prevent or perhaps even treat AMD.

Furthermore, though there is substantial knowledge as to the nature and function of carotenoids in living systems, little is known about the evolutionary history of such molecules in ocular tissues. We understand that great apes and avian species accumulate these vitamins in the retina, but are there other animals that also do so? The factors for which carotenoid accumulation could occur revolve around a few simple ideas. Some may be the life-span of the animal, the exposure to high energy light rays, and dependence on visual performance. My lab has allowed me to investigate this further and preliminary work has been done to obtain eye samples from several different species to test this hypothesis. Further work would revolve around the activity of BCO cleavage enzymes and homologues of the human GSTP 1 and StARD3 binding proteins in the several species being tested.

Overall, my experience with the Bernstein lab has been overwhelmingly positive. As mentioned, there were many activities that were extremely tedious and comparable to chores in my mind. However, the excitement of discovery and progress is addictive. I am now highly motivated to pursue some of my own hypotheses as a consequence of my experience. Considering the extreme rigor that is required to perform good science, I also have a new-found respect for

laboratorial sciences. Though I leave with a firmer conviction that working with people is more personally desirable than working with mice and protein gels, I also leave with increased curiosity. Seeing that I will likely not pursue a doctoral degree in research, this may not become part of my life for an extended period of time. However, the drive to discover will continue into my future clinical work as a physician. I am convinced that I won't ever stop asking why.

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Supplementation with macular carotenoids improves visual performance of transgenic mice



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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.

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 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

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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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 transgene 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–10 °C for 30 min 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 2000 \times g for 5 min 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 \times g for 10 min, and the supernatant was injected into the HPLC system. HPLC separations were performed on a silica-based nitrile bonded column (25 cm length \times 4.6 mm internal diameter; 5- μ m 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, Lethbridge, 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°/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 (Invitrogen, Carlsbad, CA). PCR to detect the expression of the human *GSTP1* transgene was performed with 1 μ l 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 μ m 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

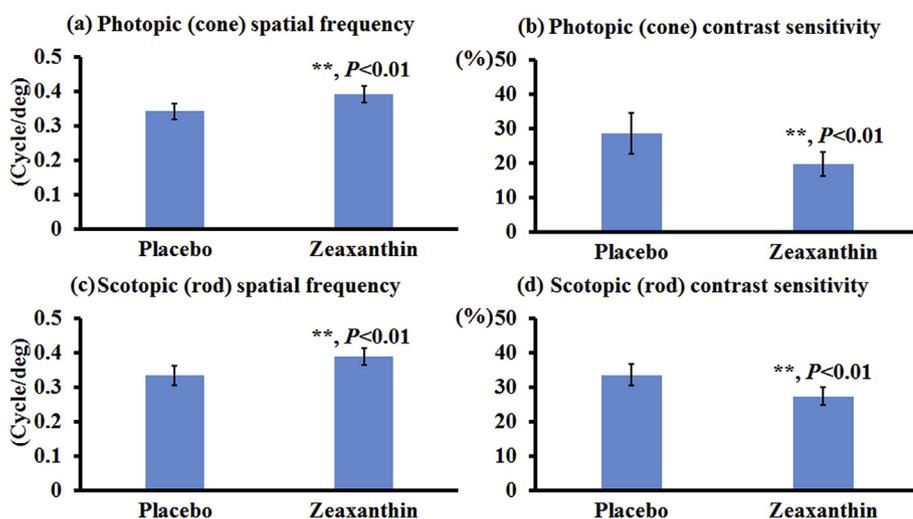


Fig. 1. Visual performance measured by OptoMotry in *bco2*^{-/-} mice with and without zeaxanthin supplementation. Zeaxanthin supplementation significantly improves the visual function of *bco2*^{-/-} mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 10 mice were used in each group. **, *P* < 0.01.

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. Fig. 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% and 35%, respectively. Like zeaxanthin supplementation, lutein supplementation can significantly increase the visual performance of *bco2*^{-/-} mice, except for their scotopic contrast sensitivity (Fig. 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. Fig. 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 5–10% 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. Fig. 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 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. Fig. 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. Fig. 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 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 (Fig. 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

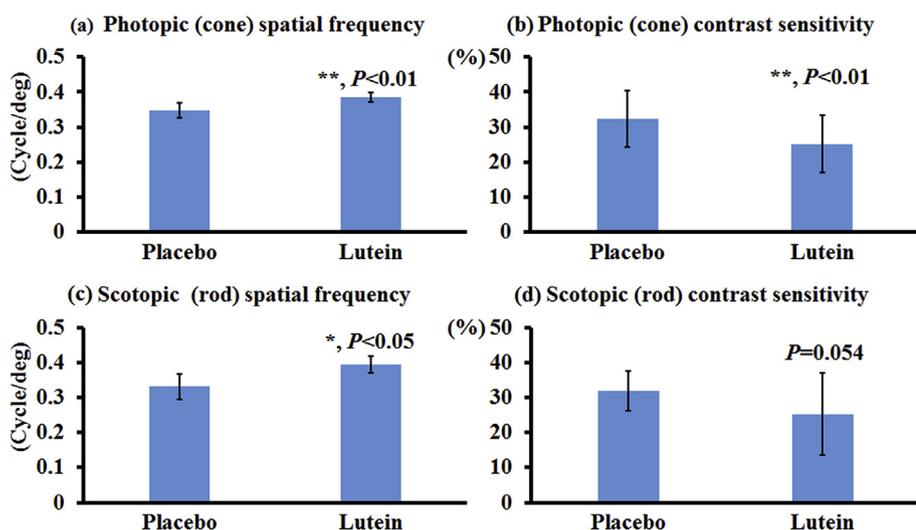


Fig. 2. Visual performance measured by OptoMotry in *bco2*^{-/-} mice with and without lutein supplementation. Lutein supplementation significantly improves *bco2*^{-/-} mice's visual function except for the contrast sensitivity of the rod cells. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means \pm SD; 15 mice were used in each group. *, *P* < 0.05; **, *P* < 0.01.

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 also shows that zeaxanthin supplementation caused a 45% increase in the cone contrast of *hGSTP1-tg/bco2*^{-/-}, which is about 1.3 times 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 (Figs. 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. (Figs. 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

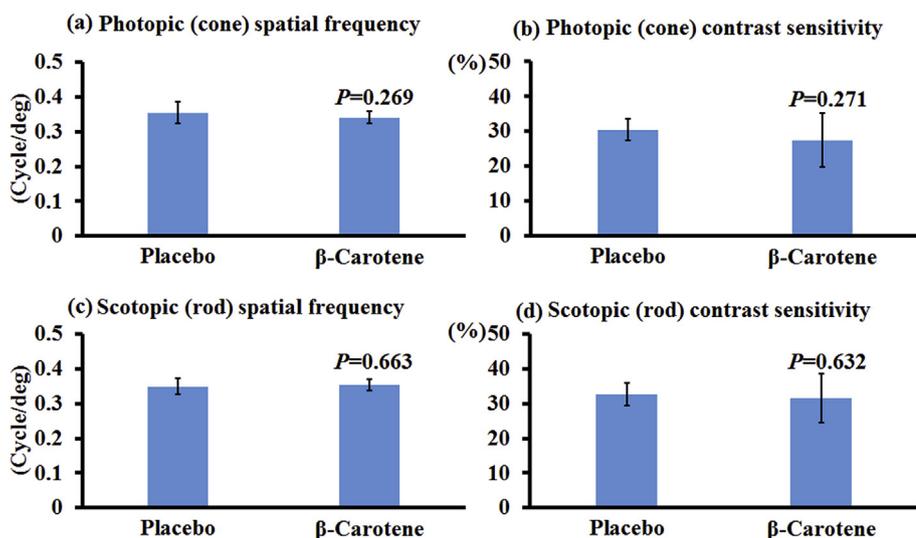


Fig. 3. Visual performance measured by OptoMotry in *bco2*^{-/-} mice with and without β -carotene supplementation. β -Carotene supplementation has no significant effect on the visual performance of *bco2*^{-/-} mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means \pm SD; 10 mice were used in each group. *, *P* < 0.05.

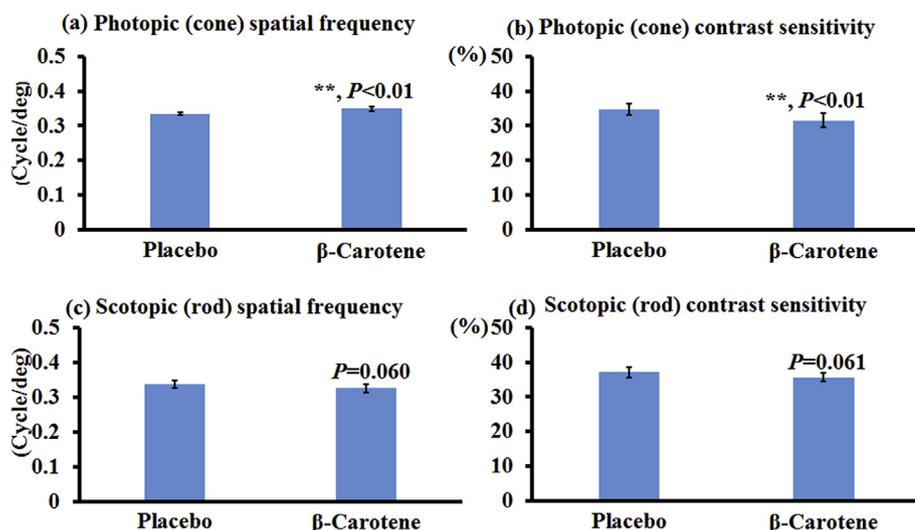


Fig. 4. Visual performance measured by OptoMotry in *bco1*^{-/-} mice with and without β-carotene supplementation. β-Carotene supplementation slightly improves the visual performance of *bco1*^{-/-} mice. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means ± SD; 7 and 8 mice were used in β-carotene and placebo groups, respectively. *, $P < 0.05$.

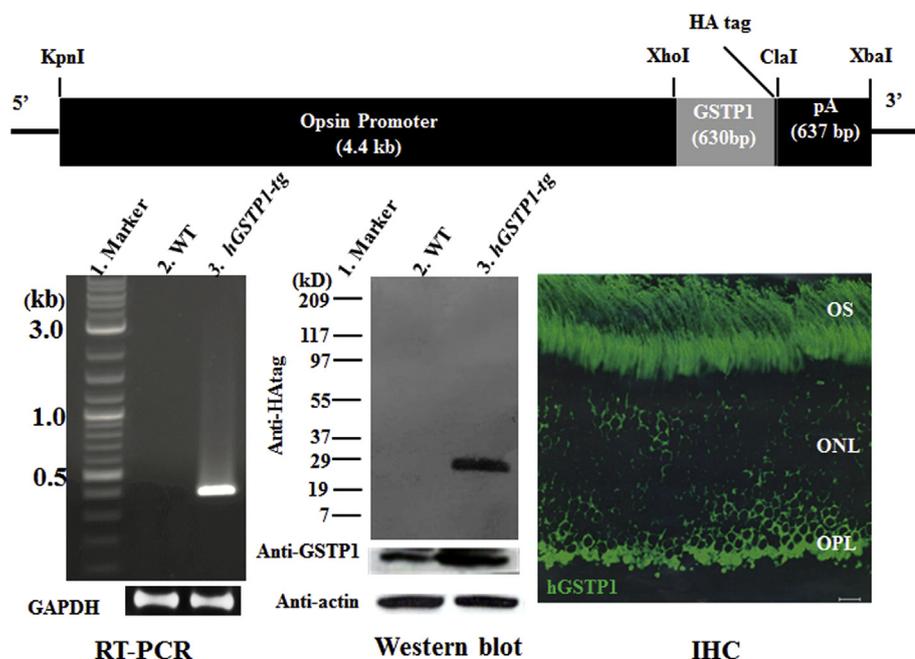


Fig. 5. Generation of transgenic (*hGSTP1-tg*) mice expressing the human zeaxanthin-binding protein GSTP1 specifically in the retina. The transgene construct (upper panel). RT-PCR reveals presence of human GSTP1 in the transgenic mouse retina. Lane 1. Amplicon size marker; 2. Wildtype C57BL/6 mice (WT); 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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 (Figs. 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 (Fig. 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) (Fig. 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 Fig. 8, if we

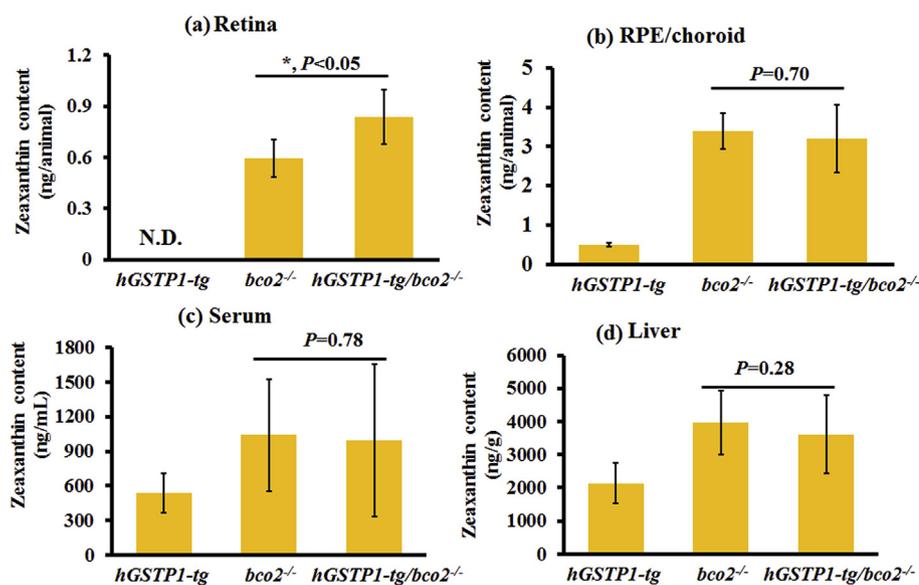


Fig. 6. Contents of zeaxanthin detected in the tissues of *hGSTP1-tg*, *bco2*^{-/-} and *hGSTP1-tg/bco2*^{-/-} mice. The expression of zeaxanthin-binding protein GSTP1 specifically in the retina of *bco2*^{-/-} mice significantly increased the retinal carotenoid contents. 8 to 10-week-old mice ($n = 25$ /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 \pm SD, N.D., not detectable. *, $P < 0.05$; **, $P < 0.01$.

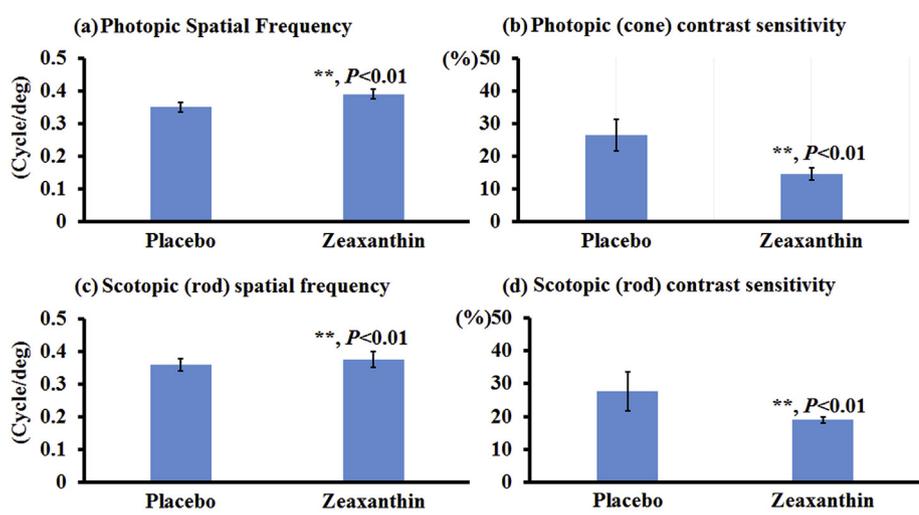


Fig. 7. Visual performance measured by OptoMotry in *hGSTP1-tg/bco2*^{-/-} mice with or without zeaxanthin supplementation. Zeaxanthin supplementation significantly improves the visual function of *hGSTP1-tg/bco2*^{-/-} mice, especially the rod contrast sensitivity. (a) Photopic spatial frequency; (b) Photopic contrast sensitivity; (c) Scotopic spatial frequency; (d) Scotopic contrast sensitivity. Values indicate means \pm SD; 11 and 14 mice were used in the zeaxanthin and placebo groups, respectively. **, $P < 0.01$.

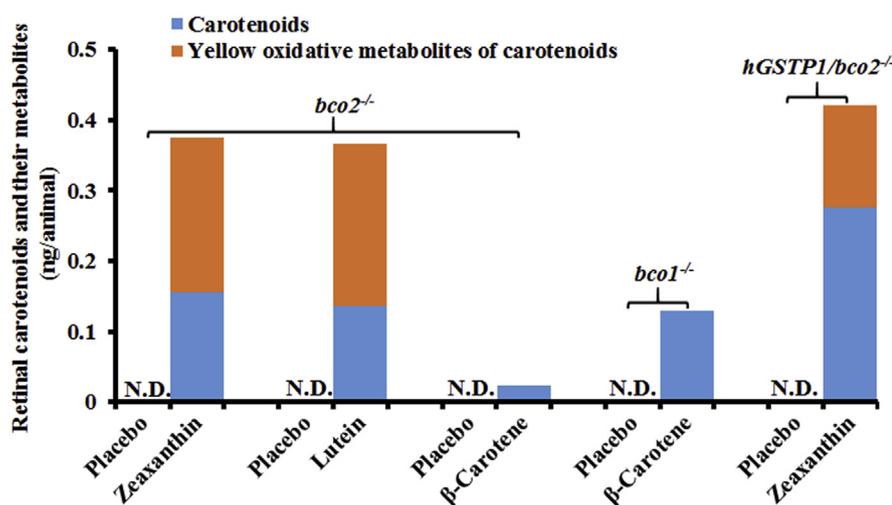


Fig. 8. Contents of carotenoids and their yellow oxidative metabolites in the retinas of the mice used in the visual performance experiments. The yellow oxidative metabolites of carotenoids were detected in the mice fed with zeaxanthin or lutein but not β -carotene, and their amounts were estimated using 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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%–30% compared to WT (*bco2*^{+/+}) mice of the same age. From the visual function data of *bco2*^{-/-} mice fed with zeaxanthin (Fig. 1), we can see that the visual function of *bco2*^{-/-} mice fed with zeaxanthin was improved 20%–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.

Conflicts of interest

The authors have no conflicts of interest.

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