Original Article

# Xanthophyll β-cryptoxanthin treatment inhibits hepatic steatosis without altering vitamin A status in β-carotene 9',10'-oxygenase knockout mice

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**Background:** β-cryptoxanthin (BCX), one of the major carotenoids detected in human circulation, can protect against the development of fatty liver disease. BCX can be metabolized through β-carotene-15,15'-oxygenase (BCO1) and β-carotene-9',10'-oxygenase (BCO2) cleavage pathways to produce both vitamin A and apo-carotenoids, respectively, which are considered important signaling molecules in a variety of biological processes. Recently, we have demonstrated that BCX treatment reduced hepatic steatosis severity and hepatic total cholesterol levels in both wide type and $BCO1^{-/-}$/$BCO2^{-/-}$ double knock out (KO) mice. Whether the protective effect of BCX is seen in single $BCO2^{-/-}$ KO mice is unclear.

**Methods:** In the present study, male $BCO2^{-/-}$ KO mice at 1 and 5 months of age were assigned to two groups by age and weight-matching as follows: (I) –BCX control diet alone (AIN-93 purified diets); (II) +BCX 10 mg (supplemented with 10 mg of BCX/kg of diet) for 3 months. At 4 and 8 months of age, hepatic steatosis and inflammatory foci were evaluated by histopathology. Retinoids and BCX concentrations in liver tissue were analyzed by high-performance liquid chromatography (HPLC). Hepatic protein expressions of SIRT1, acetylated and total FoxO1, PGC1α, and PPARα were determined by the Western blot analysis. Real-time PCR for gene expressions (*MCAD*, *SCD1*, *FAS*, *TNFα*, and *IL-1β* gene expression relative to β-actin) was conducted in the liver.

**Results:** Steatosis was detected at 8 months but not at 4 months of age. Moreover, BCX supplementation significantly reduced the severity of steatosis in the livers of BCO2 KO mice, which was associated with changes in hepatic SIRT1 acetylation of FOXO1, PGC1α protein expression and PPARα protein expression in $BCO2^{-/-}$ KO mice. HPLC analysis showed that hepatic BCX was detected in BCX supplemented groups, but there were no differences in the hepatic levels of retinol and retinyl palmitate (RP) among all groups.

**Conclusions:** The present study provided experimental evidence that BCX intervention can reduce liver steatosis independent of BCO2.

**Keywords:** β-cryptoxanthin (BCX); oxygenases; fatty liver

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## Introduction

Observational epidemiological studies have indicated that increased dietary intake of carotenoids may protect against the development of several chronic and degenerative diseases, including liver diseases (1-4). Carotenoids can be grouped in provitamin A carotenoids, like α-carotene, β-carotene and β-cryptoxanthin (BCX), or non-provitamin A carotenoids, like lycopene, lutein and zeaxanthin, depending on their ability to be metabolized to the essential nutrient vitamin A. The structure of carotenoids contains a series of conjugated double bonds, which makes them susceptible to oxidative cleavage (5,6). Oxidative cleavage of carotenoids results in the formation of apo-carotenoid metabolites, important signaling molecules with biological roles different than their parent compound (7). Two major carotenoid cleavage oxygenases have been well characterized, β-carotene-15,15'-oxygenase (BCO1), which produces vitamin A, and β-carotene-9',10'-oxygenase (BCO2), which produces apocarotenoids, from both provitamin A and non-provitamin A carotenoids. Besides their role in carotenoids metabolism, recent research has attributed BCOs a more diversified role in health, with special relation to lipid metabolism (BCO1) (3). These data indicate a complex interaction between carotenoids metabolism and health, and the better understanding of this relation is of great importance.

BCX is one of the major carotenoids detected in human circulation (1,2). BCX is an oxygenated carotenoid (xanthophyll), found at high levels in citrus fruits, pumpkin and red peppers (8). BCX intake has been inversely associated with adiposity, oxidative DNA damage, lipid peroxidation and inflammation (8-13) and it is the only carotenoid whose dietary intake was inversely associated with the risk of lung cancer (14). The exact mechanism of this protective effect is unclear, since BCX can be cleaved by BCO1 and BCO2, suggesting a multifaceted biological action. A recent study from our laboratory suggested that the BCX inhibits inflammation and liver tumor development independent of BCO1/BCO2 (15,16).

Given the importance of BCO2 in carotenoid metabolism and the protective action of BCX, the aim of the present study is to evaluate the effect of BCX supplementation on hepatic steatosis and inflammation in BCO2 knockout mice and determine whether the BCX action is dependent of BCO2. We present the following article in accordance with the ARRIVE reporting checklist (available at https://hbsn.amegroups.com/article/view/10.21037/hbsn-20-404/rc).

## Methods

### *Animals, diet and experimental groups*

$BCO2^{-/-}$ knock out (KO) mice with *BCO2* gene ablation, which were provided by Dr. Johannes von Lintig (Case Western Reserve University), were used for the present study and the generation of these mice has been previously described (17). Study mice were fed to the standard laboratory chow (Harlan Laboratories, MA, USA) until experiment begin, and maintained on a 12-hour light/dark cycle in a controlled temperature and humidity room, and given AIN-93 purified diets and water ad libitum.

Two groups of male $BCO2^{-/-}$ KO mice at 1 month (n=13 in the control group; n=14 in the BCX group) and 5 months (n=5 per group) of age were assigned to two treatments by age and weight-matching for 3 months, respectively, as follows (I) –BCX [control diet (AIN-93M purified rodent diet powder, Harlan Laboratories Inc., Madison, WI, USA)]; (II) +BCX 10 mg (control diet supplemented with 10 mg of BCX/kg of diet). All study mice were given fresh diets every 2–3 days, and maintained on their respective diets until the experiment was completed. Mice in these four groups were weighed weekly and euthanized at 4 and 8 months of age, respectively, by exsanguinations under deep anesthesia without fasting. Steatosis was not observed in two groups of 4-month old mice and the objective of this study was to evaluate the effect of BCX on liver steatosis and the underlying mechanism of the BCX effect; therefore, only 8-month old mice were used to evaluate the effect of BCX supplementation on molecular mechanism in this study.

Experiments were performed under a research protocol (No. H2018-118) with approval of the Institutional Animal Care and Use Committee at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### *BCX supplementation*

BCX (>99% purity, BASF) in powder form was directly mixed with the standard AIN-93M purified rodent diet powder at the concentration of 10 mg/kg of diet, as conducted previously (12). Briefly, based on

previous information of absorption of carotenoids in rodents and humans (12,18,19), 10 mg/kg diet of BCX in this mouse study was equivalent to a daily human consumption of 0.87 mg. Specifically, as stated in our previous publication (12), the absorption of carotenoids in rodents is approximately one-seventh that in humans and a daily supplementation of 10 mg BCX/kg diet yielded a serum concentration of BCX of 9.9 μg/L in mice and the absorption of BCX in humans is approximately 8% of what is consumed (a diet containing 1,300 μg BCX yielded a serum concentration of 113 μg/L). Therefore, the dose of BCX (10 mg/kg diet) in this mouse study was equivalent to daily human intake of 0.87 mg [9.9 μg/L × 7 × (100%/8%) = 866.25 μg ≈ 0.87 mg]. This dose can be obtained from dietary citrus fruits, such as consuming three raw tangerines daily (20). Thus, the dose of BCX supplementation used here is within the physiologic range (12).

### *Histopathological evaluation of liver lesions*

Five μm sections of formalin-fixed, paraffin-embedded liver tissue were stained with hematoxylin and eosin (H&E) for histopathological examination. Two independent investigators blinded to treatment groups examined the sections under light microscopy. Hepatic steatosis was graded according to the magnitude of steatosis (both macro- and micro-vesicular fat accumulation) and the degree of liver inflammation severity as described previously (21). Briefly, the degree of steatosis was graded 0–4 (grading 0=<5%, 1=5–25%, 2=26–50%, 3=51–75%, 4=>75%), based on the average percentage area of the liver section that was occupied by fat vacuoles per field at 100× magnification under H&E staining in 20 random fields. Inflammatory foci were evaluated by the number of inflammatory cell clusters in 20 random fields at 100× magnification, which mainly constitute mononuclear inflammatory cells. Twenty fields of view at 100 magnification represent 0.63 $cm^2$, and inflammatory foci counts were represented as the number of foci per $cm^2$. A ZEISS microscope with a PixeLINK USB 2.0 (PL-B623CU) Digital Camera and PixeLINK μScope Microscopy Software was used for image capture of all histopathological examinations.

### *Protein isolation and Western blotting*

Whole-cell lysates of liver tissues were prepared as previously described (22). The following antibodies were used for Western blotting: SIRT1 (sc-74465), acetylated and total FOXO1 (sc-374427), PGC1α (sc-518025), and PPARα (sc-398394) (Santa Cruz Biotechnology, Inc.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MAB374) (Millipore). All antibodies were used according to the manufacturers' protocols. Blots were developed using the ECL Western Blotting system (Amersham) and analyzed with a densitometer (GS-710 calibrated imaging densitometer, Bio-Rad).

### *RNA extraction and quantitative real-time PCR (qRT-PCR)*

mRNA levels of *SCD1*, *FAS*, *MCAD*, *TNFα* and *IL-1β* were determined by qRT-PCR. Liver and mesenteric adipose tissue RNA was extracted using TriPure reagent (Roche Applied Science) according to the manufacturer's instructions and cDNA was synthesized using random primer Moloney murine leukemia virus reverse transcriptase (Invitrogen). qRT-PCR was performed using the SYBR Green qRT-PCR kit (Invitrogen) according to the manufacturer's instructions on an Applied Biosystems 7500 sequence detection system. For liver analysis, primer sequences were: *SCD1* forward AGAGAGAGAGGTAGCCATATC and reverse TCAAATCTCACTAATCTCTGG; *FAS* forward CCCTTGATGAAGAGGGATCA and reverse ACTCCACAGGTGGGAACAAG; *MCAD* forward GATGCATCACCCTCGTGTAAC and reverse AAGCCCTTTTCCCCTGAAG; *TNFα* forward CTGAGGTCAATCTGCCCAAGTAC and reverse CTTCACAGAGCAATGACTCCAAAG; and *IL-1β* forward AGCCAAGCTTCCTTGTGCAAGTGT and reverse GCAGCCCTTCATCTTTTGGGGTCC. For adipose tissues, primer sequences were: Acox3; UCP3; *SCD1*, *Adiponectin* forward GTGATGGCAGAGATGGCAC and reverse GCCTTGTCCTTCTTGAAGAG; for *PPARγ* forward ATCGTGGGCCGCCCTAGGCA and reverse TGGCCTTAGGGTTCAGAGGGG. Relative gene expression adjusted to β-actin was determined using the $2^{-\Delta\Delta CT}$ method as described previously (23), and expressed as fold change. *β-actin* sequence primer sequences were forward ACGGCCAGGTCATCACTATTG and reverse TGGAAAAGAGCCTCAGGGC.

### *High-performance liquid chromatography (HPLC)*

The livers samples for HPLC analysis were prepared

**Table 1** BW and hepatic concentrations of BCX and retinoids (retinol and RP) in BCO2-KO mice at 4 and 8 months old that were fed with the control or BCX diet for 3 months

| Group | 4 months old | | 8 months old | |
|---|---|---|---|---|
| | –BCX (n=13) | +BCX 10 mg/kg diet (n=14) | –BCX (n=5) | +BCX 10 mg/kg diet (n=5) |
| Initial BW (g) | 17.0±1.6 | 17.0±1.6 | 23.1±2.0 | 21.8±0.9 |
| Final BW (g) | 23.3±2.1 | 23.9±2.9 | 28.9±4.6 | 28.5±2.0 |
| BCX (nmol/g) | ND | 0.54±0.12[a] | ND | 0.64±0.18[a] |
| Retinol (nmol/g) | 25.2±10.6 | 27.9±13.4 | 26.7±12.4 | 31.2±16.9 |
| RP (nmol/g) | 4,161±815 | 4,295±560 | 4,185±823 | 4,340±370 |
| TG (mg/g) | 15.2±8.6[a] | 12.9±7.4[a] | 30.7±6.4[b] | 12.2±6.9[a] |

Values are expressed as means ± SD (ANOVA overall F-test followed by Tukey's honest test). [a], For a given row, under the same age group, data not sharing a common superscript letter are statistically significantly different at P≤0.05. BCX, β-cryptoxanthin; BCO2, β-carotene-9',10'-oxygenase; KO, knock out; BW, body weight; RP, retinyl palmitate; TG, triglycerides; ND, not detected; SD, standard deviation.

as previously described (10,12). Specifically, a total of 0.1 g of tissue (wet weight) was homogenized (30 seconds) with internal standards [echinenone (50 µL) and retinyl acetate (50 µL)] and 1 mL of normal saline (1 minute). After the addition of 5 mL of $CHCl_3$/$CH_3OH$ (2:1, v/v), the mixture was homogenized again (30 seconds) and then vortexed (1 minute) and centrifuged for 10 minutes at 800 ×g at 4 ℃. After the lower layer of $CHCl_3$ was collected, hexane (5 mL) was added to the remaining sample; the mixture was again mixed on a vortex (1 minute) and centrifuged for another 10 minutes at 2,000 ×g at 4 ℃. The upper layer of hexane was collected. The chloroform and hexane layers were combined and evaporated under N2, after which they were reconstituted with 50 µL of ethanol and ether (2:1). A 50-µL aliquot of the extract reconstituted was injected onto the HPLC. A gradient reverse-phase HPLC system consisting of a Waters 2695 separation module and a Waters 2998 photodiode array detector was used for the detection of BCX, retinol, and retinyl palmitate (RP). Briefly, BCX, retinol, and RP were analyzed on a reverse-phase C18 column (4.6×250 mm, 5 mm; Vydac 201TP54, Grace Discovery Sciences, Inc.) with a flow rate of 1.00 mL/min and quantified relative to an internal standard by determining the peak areas against known amounts of standards. BCX and retinoids (retinol and RP) were identified by co-elution with standards and quantified relative to the internal standards (echinenone for BCX and retinyl acetate for retinol and RP), by determining peak areas calibrated against known amounts of standards.

### *Statistical analysis*

Data were presented as mean ± standard deviation (SD) for animal weights, liver inflammatory foci, liver BCX and retinoids concentrations, or as mean ± standard error of the mean (SEM) for mRNA and protein levels, and statistical differences among groups were determined by one-way ANOVA. Steatosis grade (ordinal variable) was presented as medians (grading range), and Kruskal-Wallis overall test followed by Wilcoxon rank-sum test was used to test for statistical significance among groups. SAS, version 9.2 (SAS Institute, Cary, NC, USA) was used for all statistical analyses. All P values were set at the significance level of $\alpha$=0.05 (P value ≤0.05).

## Results

### *Body weights (BWs) and concentrations of BCX and retinoids*

BWs did not differ between groups at the same age at the beginning or end of the study (*Table 1*). After 3 months of treatment, hepatic retinol and RP did not differ between groups. BCX was not detected in the liver of –BCX group, but was detected after BCX supplementation in the +BCX 10 mg groups. BCX supplementation at the dose of 10 mg/kg of diet significantly increased hepatic BCX concentrations as compared with the –BCX group (*Table 1*).

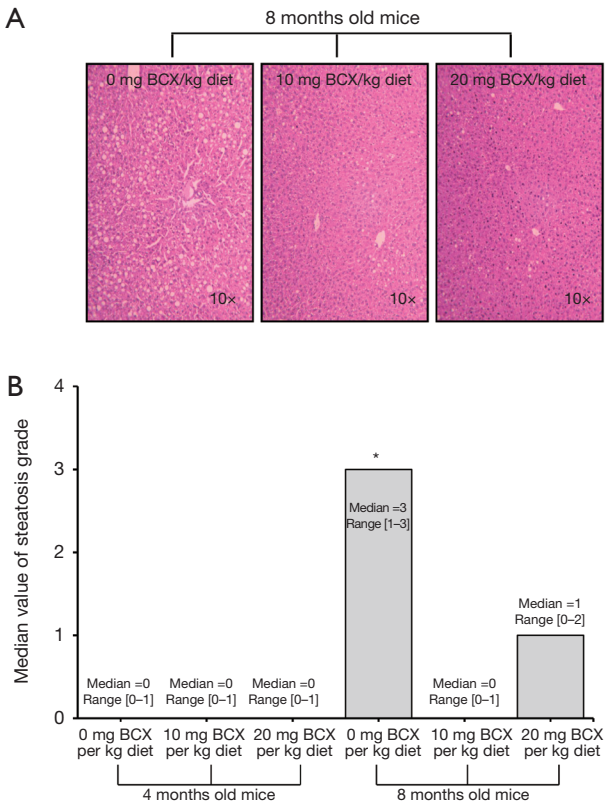


**Figure 1** Representative H&E stained sections of livers of hepatic steatosis in BCO2-KO mice at 8 months old fed control or BCX diet (A) and the comparison of median of steatosis score among groups (B). *, median score differed from that of the –BCX group, P≤0.05. H&E, hematoxylin and eosin; BCO2, β-carotene-9',10'-oxygenase; KO, knock out; BCX, β-cryptoxanthin.

### *Hepatic steatosis and inflammatory foci*

Severe hepatic steatosis was observed in the BCO2$^{-/-}$ KO mice at 8 months old without BCX supplementation as compared with that of BCX supplemented groups (*Figure 1A*). The steatosis ranged from grade 1 to 3 with the median value of 3 and no mice had grade 0 in the –BCX group (*Figure 1B*). BCX supplementation significantly reduced steatosis. In the +BCX 10 mg group, the steatosis grade ranged from 0 to 1 with the median value of 0 and no mice had grade 2 or 3 of steatosis score (*Figure 1B*). Similarly, BCX supplementation also significantly reduced hepatic levels of triglycerides (TG) at 8 months (*Table 1*). Hepatic inflammatory foci as represented in *Figure 2A* were detected in all groups, and inflammatory foci were lower in the BCX supplemented group, though the differences were not statistically significant (*Figure 2B*).

### *Hepatic SIRT1 expression and activity, PGC1 and PPAR expression*

Hepatic SIRT1 protein expression was not different between the groups (*Figure 3A*). Acetylated FOXO1 expression, a well-known target of SIRT1, was significantly lower in the +BCX group after BCX supplementation as compared with the –BCX group, indicating that hepatic SIRT1 deacetylase activity was induced by BCX (*Figure 3B*). Hepatic PGC1α protein expression, another SIRT1 target, was higher in the +BCX group in comparison with the –BCX group (*Figure 3C*). Hepatic PPARα protein expression was also higher in the +BCX group as compared with the –BCX group (*Figure 3D*).

### *Expression of genes involved in lipid metabolism and inflammation*

The hepatic *FAS* and *SCD1* gene expressions were lower in the +BCX group (*Figure 4A,B*), and the hepatic gene expression of *MCAD* was higher in the +BCX group (*Figure 4C*) in comparison to the –BCX group. No differences in the hepatic *TNF*α and *IL-1*β mRNA expression were observed between the groups (data not shown). Mesenteric adipose tissue gene expression for *Adiponectin*, *Acox3*, and *SCD1* did not differ between the groups (data not shown). For *PPAR*γ in the mesenteric adipose tissue, BCX treatment marginally increased the gene expression in the +BCX group in comparison to the –BCX group (*Figure 4D*).

## Discussion

The present study shows that BCO2$^{-/-}$ KO mice fed with a vitamin A sufficient diet [AIN-93M purified diet (% kcal from carbohydrate, protein and fat: 76%, 13.8% and 10.2%, respectively)] without carotenoid supplementation developed liver steatosis at 8 months old but not at 4 months old. This finding suggests that the occurrence of steatosis is related to age of the mice in the BCO2$^{-/-}$ KO mice, which is consistent with the result from the study by Hessel *et al.* (24) that studied BCO1 knockout mice with different ages and found that BCO1 deficient mice developed fatty liver independent of the vitamin A or carotenoids content of the diet. The authors also described that steatosis was more pronounced with age (24), supporting our findings that steatosis was only detected in mice with 8 months old, and not at 4 months old. In fact,

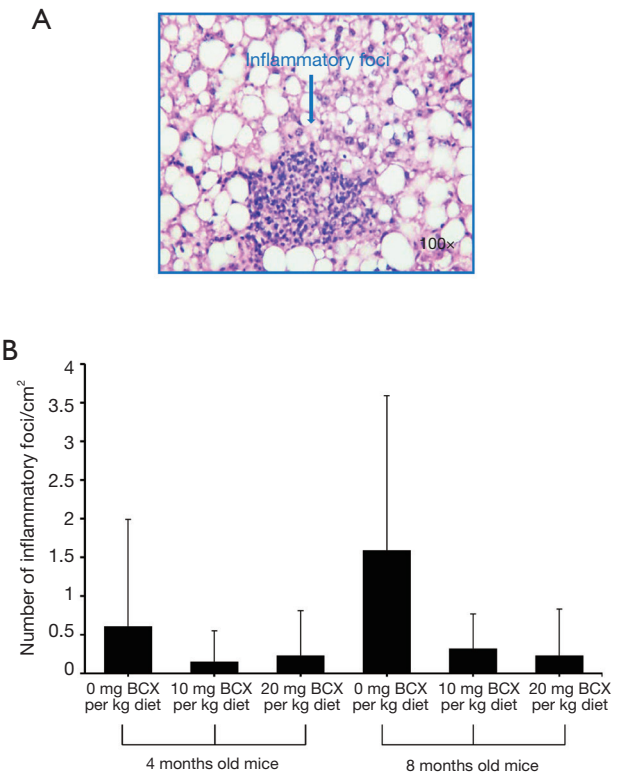


**Figure 2** Insert: representative inflammatory foci as seen on a H&E stained slide (A) and the number of inflammatory foci in BCO2-KO mice at 4 and 8 months old fed control or BCX diet (B). Values are mean ± SD. H&E, hematoxylin and eosin; BCO2, β-carotene-9',10'-oxygenase; KO, knock out; BCX, β-cryptoxanthin; SD, standard deviation.

aging has been associated with increased accumulation of lipids in non-adipose tissue organs, like the liver (25). Furthermore, our finding in BCO2$^{-/-}$ KO mice aged 4 months old is also consistent with the result from the study by Amengual *et al.* (17) described no steatosis was observed in BCO2$^{-/-}$ KO mice at ~3 months (13 weeks) of age, that were fed standard chow diet (60%, 28%, and 12% of the % kcal from carbohydrate, protein, and fat, respectively) without carotenoid supplementation. These data suggest that despite the different diets used in these two studies, BCO2$^{-/-}$ KO mice aged 3–4 months old did not developed steatosis. The liver steatosis developed in BCO2$^{-/-}$ KO mice aged 8 months old could be in part due to age of animals. However, due to the lack of wild type of mice, we cannot exclude the possibility of steatosis development was due to BCO2 KO. A recent study that showed that BCO2 genotype affects mitochondrial functioning and energy homeostasis (26) provided the evidence that the BCO2 genotype may play a role in liver steatosis. But, importantly, in this study, BCX supplementation significantly reduced the severity of liver steatosis in the BCO2$^{-/-}$ KO mice with no effect on hepatic retinoid levels. Retinoids have been associated with improved steatosis and metabolism regulation in the liver (27-29). Since apo-carotenoid, metabolites of BCO2 can be further cleaved by BCO1 to generating vitamin A (30), our findings suggest that BCX itself exerted a protective effect. This is corroborated by our recent study with BCX supplementation in fatty liver and hepatocellular carcinoma in mice, in which BCX was effective in preventing those outcomes (12,15,16,31). By contrast, in the study by Amengual *et al.* (17), severe liver steatosis was developed in BCO2-KO mice at ~3 months (13 weeks) of age after feeding a vitamin A-deficient purified diet (based on AIN-93G formulation) supplemented with 50 mg/kg lutein or 50 mg/kg zeaxanthin. The authors attribute liver steatosis to hepatic mitochondria stress and dysfunction due to the accumulation of carotenoids metabolites (17). Despite the same genetic background, the dose used in the study by Amengual *et al.* (17) was 5 times higher than the dose used in our study, and carotenoid used was also different. Ford *et al.* (32) studied hepatic lipid accumulation in wild type, BCO1 and BCO2 knockout female mice at 7 months old fed with chow diet, lycopene supplemented diet or tomato powder supplemented diet and found that BCO2 knockout mice displayed higher lipids content in the liver, but not steatosis, in comparison to wild type mice, independently of the diet consumed. The authors also evaluated inflammatory markers, but found no differences between groups (32). Taken together, these data suggest that impaired lipid metabolism may explain the presence of steatosis observed in the present study. Additionally, no effect of BCX supplementation on retinoid levels in this study confirms recent reports that showed that BCX is BCO2-dependently converted to retinoids and that BCO2 is the major BCX metabolizing enzyme in mice (33).

It is also important to highlight that the BCX dose used in this study was relevant to physiologic levels. First, data from our previous study showed that the detected BCX serum concentration in A/J mice supplemented with 10 mg BCX/kg diet was lower than that of the average U.S. population (12). Second, the mean liver BCX concentration in the mice supplemented with 10 mg BCX/kg diet in this study (0.64 nmol/g) was comparable to that reported in human livers (0.66 nmol/g) (34). Furthermore, the BCX

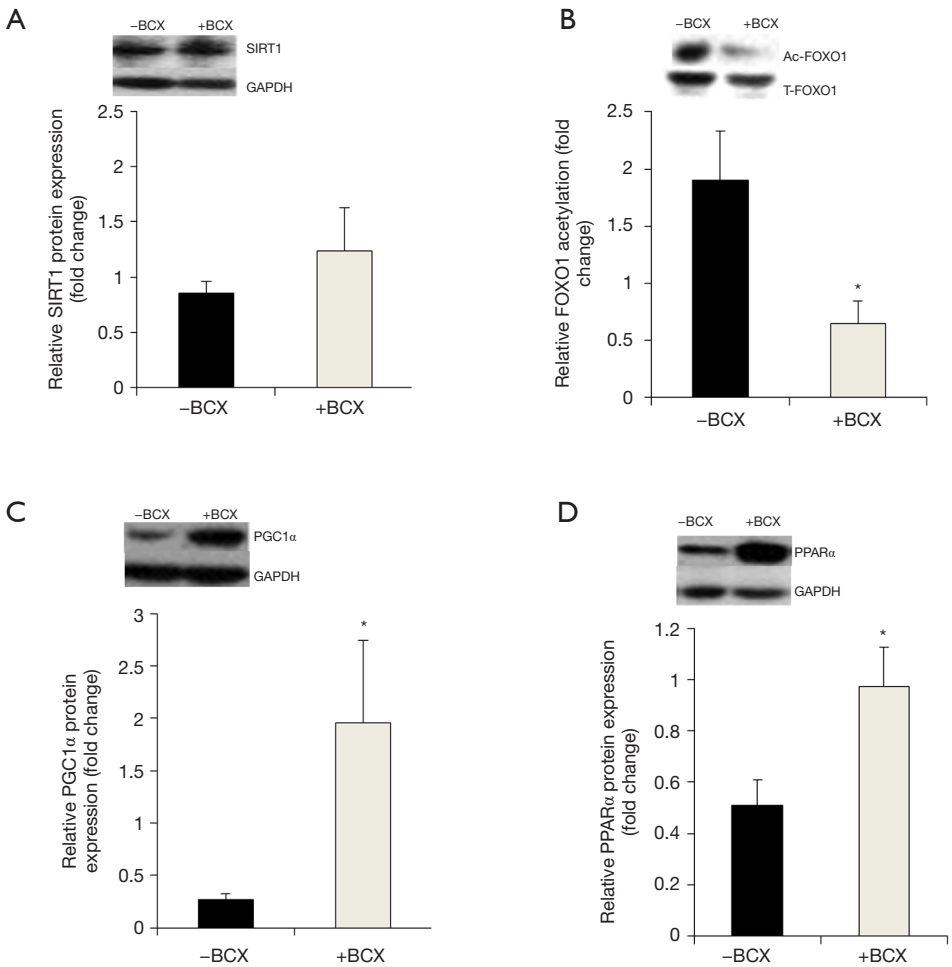


**Figure 3** Hepatic SRT1 protein expression (A), acetylation of FOXO1 (B), PGC1α protein expression (C), and (D) PPARα protein expression in BCO2-KO mice fed control or BCX diet for 3 months. Values are mean ± SEM, n=5. *, different from –BCX, P≤0.05. BCO2, β-carotene-9',10'-oxygenase; KO, knock out; BCX, β-cryptoxanthin; SEM, standard error of the mean.

dose used in the present study, which was equivalent to daily human consumption of 0.87 mg of BCX, can be obtained from dietary citrus fruits, such as consuming three raw tangerines daily (15,16,31).

In the present study, the significant reduction in hepatic fat accumulation by BCX was associated with the induction of SIRT1 activity and upregulation of PGC1α and PPARα in the liver tissue of BCO2-KO mice. BCX significantly decreased the levels of acetylated FOXO1, indicating an increase in SIRT1 enzyme activity by BCX supplementation. We and others have shown that SIRT1 activation can protect liver against fat accumulation (35-38). The mechanisms by which SIRT1 activation may attenuate steatosis include the deacetylation and inactivation of SREBP-1c and the promotion of deacetylation and activation of PGC1α (39,40). Hepatic lipogenesis is mainly regulated by SREBP-1c, which increases the expression of lipogenic genes such as *FAS* and *SCD1*. FAS is a rate-limiting enzyme in the fatty acid biosynthesis and catalyzes the last step of *de novo* lipogenesis, and SCD1 catalyzes the formation of monounsaturated long-chain fatty acids. Previously, the depletion of SCD1 in obese mice resulted in the reversal of hepatic steatosis (41). In this context, increased SIRT1 activity can lead to SREBP-1c downregulation, suppressing lipogenic genes expression, contributing to reducing hepatic steatosis (42). On the other hand, the activation of PGC1α may increase the PPARα-mediated gene expression, like fatty acid oxidizing enzymes

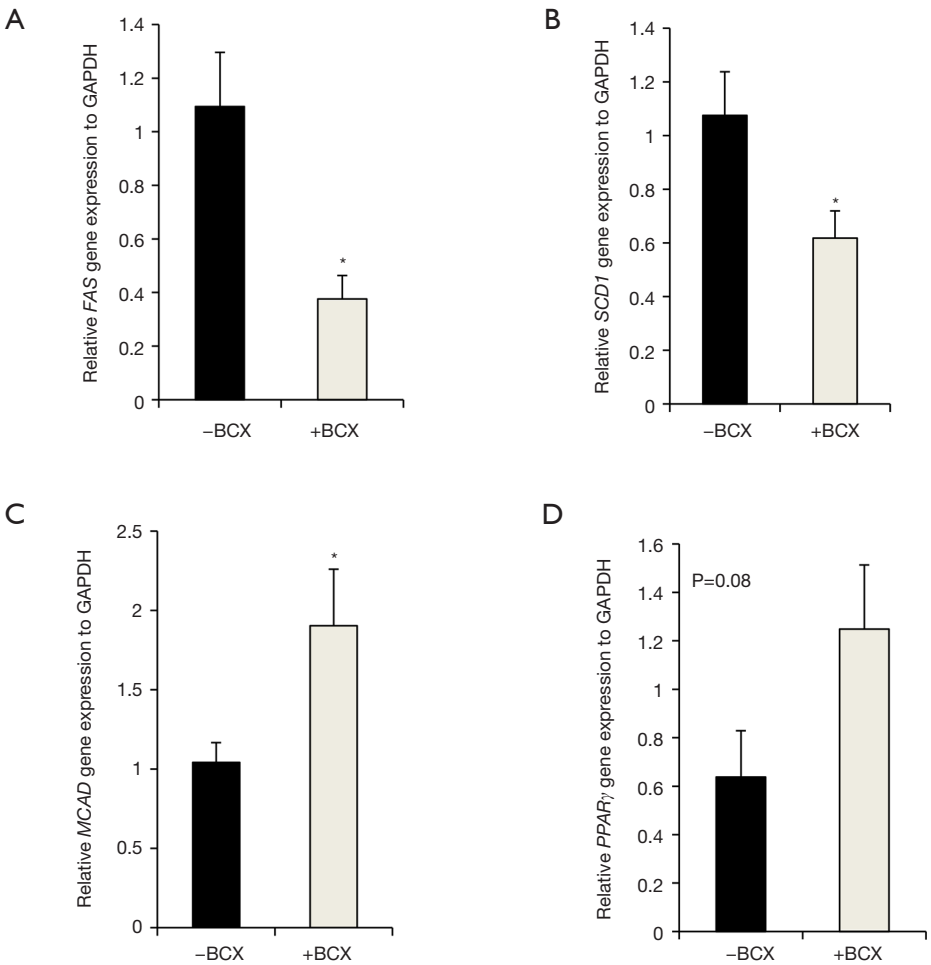


**Figure 4** Hepatic *FAS* mRNA (A), *SCD1* mRNA (B), *MCAD* mRNA (C), and adipose tissue *PPARγ* mRNA (D) in BCO2-KO mice fed control or BCX diet for 3 months. Values are mean ± SEM, n=5. *, different from –BCX, P≤0.05. BCO2, β-carotene-9',10'-oxygenase; KO, knock out; BCX, β-cryptoxanthin; SEM, standard error of the mean.

such as very long-chain (ACADVL) and MCAD (43-45). These dehydrogenases are involved in the first step of the β-oxidation, and are responsible for the dehydrogenation of the acyl-CoA ester. Indeed, the deficiency of MCAD and ACADVL was associated with hepatic steatosis in mice, supporting their role in the fatty acid β-oxidation in the liver (41). In the present study, BCX increased SIRT1 activity/PGC1α-PPARα expression, which was associated with both inhibition of fatty acid synthesis genes FAS and SCD1 and up-regulation of fatty acid oxidation gene MCAD in the liver of BCO2-KO mice. In the mesenteric adipose tissue, no difference in lipid metabolism markers was observed. PPARγ in adipose tissue was marginally increased after BCX treatment. The activation of PPARγ stimulates the storage of fatty acids in mature adipocytes, contributing to reduced lipotoxicity (46). Taken together, these data suggest that BCX itself protects against the development of steatosis in BCO2-KO mice by targeting hepatic SIRT1 activity and downregulating hepatic lipid synthesis, and upregulating hepatic fatty acid oxidation. The upregulation of SIRT1 activity and PPARα expression is in accordance with previous studies (11,12,47). However, the mechanism by which BCX increases SIRT1 remains to be further explored.

In summary, BCX itself induced hepatic SIRT1 activity, activating SIRT1/PGC1α/PPARα signaling pathway and inhibiting hepatic lipogenesis that ultimately led to decreased hepatic steatosis. The present study raised

important points about gene-diet interaction between the BCO2 enzyme and dietary carotenoids. First, it supports previous observations that the aging process is related to lipid metabolism alterations in the liver. Second, given that the single-nucleotide polymorphism (SNP) in the *BCO2* gene has been reported and associated metabolic alterations in humans (3), the present study suggests that BCX at physiologic levels may exert biological function and activate SIRT1 pathways in the absence of BCO2.

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## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://hbsn.amegroups.com/article/view/10.21037/hbsn-20-404/rc

*Data Sharing Statement:* Available at https://hbsn.amegroups.com/article/view/10.21037/hbsn-20-404/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://hbsn.amegroups.com/article/view/10.21037/hbsn-20-404/coif). XDW serves as the unpaid Deputy Editor-in-Chief of *Hepatobiliary Surgery and Nutrition*. The other authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a research protocol (No. H2018-118) with approval of the Institutional Animal Care and Use Committee at the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.



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