Zeaxanthin Exhibits Protective Effects in Myocardial Injury by Inhibiting TGF-β/Smad2/3 and p38MAPK/NF-κB Signaling Pathways

Mingyang Li
The Fourth Affiliated Hospital of Harbin Medical University

Xiang Song
The Hospital of Zhou Pu

Lichun Qi
The Fourth Affiliated Hospital of Harbin Medical University

Yanhui Gao
The Fourth Affiliated Hospital of Harbin Medical University

Xin Wang
The Fourth Affiliated Hospital of Harbin Medical University

Xiao Zhong
The Fourth Affiliated Hospital of Harbin Medical University

Ziguang Song
The Fourth Affiliated Hospital of Harbin Medical University

Xueqi Li (✉ is9150@163.com)
The Fourth Affiliated Hospital of Harbin Medical University

Research Article

Keywords: cardiomyocyte apoptosis, HFD, myocardial injury, myocardial fibrosis, zeaxanthin

Posted Date: October 27th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-966160/v1

License: ☺️ ☀️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background: Zeaxanthin is a newly discovered natural product in β-carotenoid family with multiple bioactivities. Recently, it has been shown that zeaxanthin may have cardioprotective effects in several studies, but its mechanisms have not been fully investigated. Herein, we explored the role and mechanism of zeaxanthin in myocardial injury.

Methods and Results: In this study, three different models were used to investigate the mechanism by which zeaxanthin alleviates myocardial injury. H9C2 Cardiomyocyte injury models were induced by H$_2$O$_2$. TUNEL assay, Flow cytometry, and Western blot analysis showed that treatment with zeaxanthin significantly decreased cardiomyocyte apoptosis and apoptosis-related protein expression. And reactive oxygen species (ROS) measurement analysis and Western blot analysis showed that treatment with zeaxanthin also could reduce the production of ROS and affect the expression of p38-Mitogen activated protein kinase/nuclear factor-κ gene bindin (p38MAPK/NF-κB) signaling pathway. Transforming Growth Factor-β1 (TGF-β1) was used to establish the fibrosis model in cardiac fibroblasts (CFs). QRT-PCR and Western blot analysis showed that treatment with zeaxanthin significantly decreased the expression of fibrosis markers in CFs. Myocardial injury animal models were induced by high-fat diet (HFD). Our results demonstrated that zeaxanthin improved fibrosis damage and cardiomyocyte apoptosis in HFD mice. Furthermore, Western blot analysis showed that TGF-β/Drosophila mothers against decapentaplegic2/3 (TGF-β/Smad2/3) signaling pathway related protein p-Smad2/3, Smad2/3, and TGF-β1 were significantly downregulated by zeaxanthin treatment.

Conclusions: Zeaxanthin may alleviate HFD and H$_2$O$_2$-induced heart injury by regulating TGF-β/Smad2/3 and p38MAPK/NF-κB signaling pathways, which is of immense clinical significance in the treatment of cardiovascular disease.

Background

Myocardial fibrosis and cardiomyocyte apoptosis are the predominant manifestations of pathological changes associated with myocardial injury. When myocardial injury occurs, it induces cardiomyocyte apoptosis by affecting the expression of apoptosis-related signaling pathways and genes. And it also leads to the activation of CFs, followed by their transdifferentiation into myofibroblasts. Subsequently, scar tissue formed by extracellular matrix (ECM) deposition replaces normal myocardial tissue, increases ventricular wall stiffness, resulting in decreased cardiac compliance, cardiac dysfunction, and eventually heart failure [1]. Myocardial injury is closely related to the occurrence and development of a range of cardiovascular diseases, such as hypertension, heart failure, myocardial hypertrophy, and arrhythmia [2–4]. However, insights into the mechanism underlying myocardial injury remain scarce, although such mechanistic would be helpful in preventing cardiac dysfunction and identifying therapeutic targets for clinical treatment [5, 6]. Therefore, identifying targets and effective therapeutic drugs for myocardial injury have gained substantial clinical significance.
Zeaxanthin is a natural fat-soluble isoprene compound commonly. It has many pharmacologically beneficial effects, such as antioxidative, antiinflammatory, anticancer, and anti-aging activities, reduction of blood lipids, and prevention of cataracts. Zeaxanthin has been shown to downregulate the expression of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, and serum bilirubin in a dose-dependent manner, thereby alleviating liver fibrosis [7]. And zeaxanthin significantly inhibits the progression of nonalcoholic steatohepatitis by reducing oxidative stress and liver fibrosis [8]. Zeaxanthin has been shown to exert a protective effect on cardiovascular function and reduce heart failure induced by doxorubicin [9]. In addition, zeaxanthin can also stall disease progression in patients with congestive heart failure in a dose-dependent manner [10]. A mixture of antioxidants, including zeaxanthin, astaxanthin, and lutein, was demonstrated to protect the myocardium from ischemia/reperfusion injury by reducing oxidative stress and apoptosis, and has a certain beneficial effect in the treatment of cardiovascular complications [11]. Zeaxanthin and carotenoids may also reduce the risk of coronary heart disease on account of their antioxidant properties [12, 13].

However, the regulatory effect of zeaxanthin on myocardial injury, along with the underlying mechanism of action, has not yet been reported. Therefore, our study will explore the role and mechanism of zeaxanthin in myocardial injury for the first time.

Materials And Methods

Material

Zeaxanthin was purchased from Zelong Biotechnology (Shanxi China) and stored at −20 °C. It was dissolved in DMSO for in vitro and in vivo experiments. High-fat diet (HFD: protein 18.1%, fat 61.6%, carbohydrate 20.3%) and normal diet (ND: protein 18.3%, fat 10.2%, carbohydrate 71.5%) were purchased from Trofe Feed Technology (Jiangsu, China).

Animal model

All animal experiments were performed according to the principles and guidance of the Harbin Medical University Committee on the Use and Care of Animals. 15 Eight-week-old C57BL/6 male mice (20-25 g body weight) were used in this study and housed in SPF conditions (22 ± 2.0 °C, 50 ± 5% humidity, 12-h light/12-h dark cycle, free access to water and food). To establish the myocardial injury model using HFD, mice were divided into three groups: ND, HFD, and HFD + Zeaxanthin. Zeaxanthin was given by oral gavage at a dose of 100 mg/kg once a day. ND and HFD groups were gavage with vehicle only. Mice were sacrificed after 8 weeks of feeding. All animals were euthanized with CO2 and ventricular tissues were harvested and stored at -80 °C for further experiments.

Cardiomyocyte culture

H9C2 cardiomyocytes were purchased from the Cell Bank of Chinese Academy of Sciences. H9C2 cardiomyocytes were maintained in DMEM containing 10% FBS and 1% penicillin-streptomycin and
cultured in a standard humidified incubator at 37 °C with 5% CO2. After starvation in serum-free medium for 12 h, cardiomyocytes were treated with zeaxanthin (60 µM) for 24 h and stimulated with H2O2 (200 µM) for 12 h. And in the H9C2 cardiomyocyte experiments, the ctrl groups were untreated as the blank control. At the end of the experimental period, all used materials were treated innocuously.

CF culture

Primary CFs were isolated from the heart tissue of Kunming mice. CFs were cultured in DMEM with 10% FBS and 1% penicillin-streptomycin and then placed in an incubator maintained at 37 °C with 5% CO2 and 95% air. Before TGF-β1 treatment, CFs were cultured in a serum-free medium for 6 h. The concentration of TGF-β1 used was 10 ng/mL. CFs were stimulated for 24 h with TGF-β1 and treated with zeaxanthin for 24 h, followed by extraction of RNA or protein for further analysis. In the CF experiments, the ctrl groups were untreated as the blank control. At the end of the experimental period, all used materials were treated innocuously.

Flow cytometry

An Annexin V-FITC/PI apoptosis detection kit (Solarbio, Beijing, China) was used to check for apoptosis in H9C2 cells. After the cells were grown and subjected to the appropriate treatment, an appropriate amount of cells were collected in the logarithmic growth phase and washed twice with PBS. The harvested cells were then incubated with a buffer solution containing annexin V-FITC for 10 min, followed by treatment with PI for another 10 min at room temperature. Flow cytometry was used to measure cellular apoptosis levels.

ROS measurement

A DCFH-DA (10 µM) probe was used to detect ROS formation in cardiomyocytes. Cells were incubated with a probe for 30 min at 37 °C. The culture medium was discarded, and cells were fixed with 4% paraformaldehyde, followed by three steps of washing with PBS. Images were acquired using a fluorescence microscope and analyzed using ImageJ.

Blood lipid measurement

Serum from animals in each group was collected and centrifuged at 3000 rpm for 10 min. The resultant supernatant was directly monitored using an automatic biochemical instrument (Rayto, Shenzhen, China). Triglyceride (TG) (C061-a), total cholesterol (TC) (C063-a), and low-density lipoprotein (LDL) (C062-b) standards were obtained from Huili Biotechnology (Changchun, China).

H&E and Masson staining

The ventricular tissues of 3 mice in each group were taken out for experiment. Myocardial tissues were fixed with 4% paraformaldehyde at 4 °C for 24 h. Samples were dehydrated to transparency, and paraffin embedding was performed. The embedded tissue was cut into 5-µm thick slices using a paraffin slicer
and fixed on an adhesive slide. Sections were then stained using a Hematoxylin-Eosin (H&E) staining kit (Solarbio, Shanghai, China). Masson's trichrome staining kit (Solarbio, Shanghai, China) was used to examine collagen deposition in myocardial tissues.

**Immunofluorescence assay**

The ventricular tissues of 3 mice in each group were taken out for experiment. The paraffin-embedded myocardial tissue was sectioned into slices of 5-μm thickness. Sections were dewaxed and rehydrated, and permeabilized by treatment with 0.1% Triton X-100 (4 μL Triton X-100 and 0.1 g BSA in 1 mL PBS) for 1 h. Subsequently, the permeabilized sections were blocked with 50 % normal goat serum at 37 °C for 1 h. Then, 50 μL of rabbit anti-Bax primary antibody (1:100, Abcam) was added to each tissue section and incubated overnight at 4 °C. The primary antibody was recovered the next day. Tissue sections were washed with PBS, followed by incubation with a goat anti-rabbit secondary antibody labeled with FITC (1:500, Alexa Fluor 594, Life Technology) at room temperature for 1 h. Nuclei were stained with DAPI for 5 min. Immunofluorescence images were acquired in a fluorescence microscope.

**MTT assay**

H9C2 cells were plated in 96-well plates, and 200 μL of culture medium was added to each well. After the appropriate treatment, 20 μL of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT) solution (5 mg/ml, Beyotime Biotechnology, China) was added to each well and cells were incubated at 37 °C for 4 h. Then, 150 μL of dimethyl sulfoxide was added to each well and incubated for 15 min. Absorbance was measured at a wavelength of 490 nm using a fluorescence microplate reader.

**CCK-8 viability assay**

Cell Counting Kit-8 (CCK-8) was used to assess the optimal time point for zeaxanthin treatment in H9C2 cells. H9C2 cells were seeded into 96-well plates. After pretreatment with 60 μM zeaxanthin for 0, 12, 24, and 48 h, H₂O₂ (200 μM) was added for 4 h to induce cardiomyocyte hypoxia. Then, the culture medium was replaced with CCK-8 solution. Absorbance was measured at 450 nm using a fluorescence microplate reader.

**TUNEL apoptosis assay**

H9C2 cells were grown to confluence after treatment and washed thrice with PBS. Cells were then treated with TUNEL solution, according to the manufacturer's instructions. TUNEL apoptosis images were obtained using an Olympus microscope. The ratio of apoptosis-positive cell nuclei/total cell nuclei represents the rate of cardiomyocyte apoptosis. Cellular apoptosis events were quantified using the Image J software.

**Western blotting**
Total proteins from cells or tissues were extracted and lysed with 40-60 μL of RIPA buffer (Beyotime Biotechnology, Jiangsu, China) containing protease inhibitors. Protein samples were separated on an SDS-PAGE gel and transferred to a pure PVDF membrane (Merck, Germany). The membrane was probed with primary antibodies overnight and incubated at 4 °C. The primary antibodies used were GADPH antibody (1:5000, No. 66535-1-lg), Fibronectin-1 (FN1) antibody (1:500, No. 15613-1-AP), Collagen-I (Col-I) antibody (1:500, No. 14695-1-AP), Bcl2-associated X protein (Bax) antibody (1:500, No. 50599-2-lg), B-cell lymphoma-2 (Bcl2) antibody (1:500, No. 12789-1-AP), cleaved-caspase-3 antibody (1:500, No. 19677-1-AP), p-NF-κB antibody (1:500, No. 66535-1-lg), p-p38MAPK antibody (1:500, No. 66234-1-lg), TGF-β1 antibody (1:500, No. 21898-1-AP), and Smad2/3 antibody (1:500, No. 12570-1-AP) (all from Proteintech, Rosemont, IL, USA). After washing with PBS, the membranes were further incubated with HRP-conjugated secondary antibodies at room temperature for 50 min. The Image Lab software was used to detect protein expression levels.

**RNA extraction and qRT-PCR assay**

Total RNA was extracted from cells using the TRIzol reagent. After TRIzol was used to lyse the cells, chloroform was added and cells were thoroughly vortexed to ensure stratification of the aqueous and organic phases. The upper aqueous phase was separated after centrifugation for 10 min and mixed gently with the same amount of isopropanol for 10 min. Afterwards, the samples were again centrifuged for 10 min at 4 °C at 12000 rpm. The supernatant was discarded and the extracted RNA was washed with ethanol. Finally, 20 μL of DEPC-treated water was added to dissolve the RNA.

A High-Capacity cDNA Reverse Transcription Kit was used to reverse transcribe the isolated RNA to cDNA. cDNA was used to detect the relative expression of mRNA on a QuantStudio 6 real-time PCR system (Applied Biosystems, Foster City, CA, USA) in the presence of 50X ROX reference dye 2 (Vazyme Biotech, Jiangsu, China). The relative mRNA expression level was calculated based on the Ct values and normalized to GAPDH levels. The primer sequences used were as follows: GAPDH (Forward:5’-ATGGGTGTGAACCACGAGA-3’, Reverse:5’-CAGGGATGATGTTCTGGGCA-3’); FN1(Forward:5’-CGAGGTGACAGAGACCACCA-3’, Reverse:5’-GACACAA CAATGCTCCAGA-3’); Col-I (Forward:5’AAGAAGACATCCCTGAAGTCA-3’, Reverse:5’-TTGTGGGAGATACAGATCAAG-3’); TGF-β1(Forward:5’-TTGCTCCACAGAGCTGAAGAGAGC-3’); Matrix-metalloproteinase-9 (MMP-9) (Forward:5’-AAAGGCAATTCAAGACCAC-3’, Reverse:5’-GGATGACAATGCTCCAG-3’); Connective tissue growth factor (CTGF) (Forward:5’-GGAAGACACATTTGGCAGAC-3’, Reverse:5’-TAGGTGTCC GGATGACACATT-3’).

**Statistical analysis**
All data were analyzed using GraphPad Prism 8.0 and expressed as mean ± SEM. The significance level was calculated by Student’s $t$-test between two groups, and one-way analysis of variance (ANOVA) was used for comparisons across multiple conditions. Bonferroni correction was used to evaluate whether there were differences in each group. A value of $p<0.05$ was considered as statistically significant.

**Results**

**Zeaxanthin suppresses cardiomyocyte apoptosis**

First, we used $H_2O_2$ to induce H9C2 cardiomyocyte injury. The CCK-8 assay was used to determine the optimal time for zeaxanthin treatment in H9C2 cells by estimating the viability of H9C2 cells after 0, 12, 24, and 48 h of exposure to zeaxanthin. Results from the CCK-8 assay showed that treatment with zeaxanthin for 24 h had the strongest effect on restoring H9C2 cell viability ($p<0.05$, **$p<0.01$). At the 48h time point, zeaxanthin had an inhibitory effect on cell viability (Fig.1a). In addition, the MTT assay was used to detect the effect of different concentrations of zeaxanthin on cell activity. Zeaxanthin was found to recover cell activity reduced by $H_2O_2$ treatment (**$p<0.01$) (Fig.1b). Through the detection of apoptosis-related markers including Bax, Bcl2, and cleaved caspase-3, western blotting confirmed that zeaxanthin at a concentration of 60 $\mu$M significantly decreased the expression of apoptosis-related markers (***$p<0.01$) (Fig.1c).

Based on these results, we selected 60 $\mu$M zeaxanthin as the optimal condition to inhibit cardiomyocyte apoptosis. TUNEL staining (Fig.2a) and flow cytometry assays (Fig.2b) showed that zeaxanthin administration could reduce H9C2 cardiomyocyte apoptosis induced by $H_2O_2$ (**$p<0.01$). Western blotting showed that zeaxanthin significantly decreased the protein expression levels of Bax, Bcl2, and cleaved caspase-3, which were increased with exposure to $H_2O_2$ ($p<0.05$) (Fig.2c).

**Zeaxanthin inhibits ROS production**

We measured the intracellular ROS level in H9C2 cells treated with H2O2 using DCFH-DA probe. As shown in Fig.2d, we found that $H_2O_2$ promoted ROS production in H9C2 cardiomyocytes, whereas zeaxanthin inhibited ROS production (**$p<0.01$). In addition, Western blotting was used to detect changes in p38MAPK/NF-$\kappa$B levels in ROS-related signaling pathways. Oxidative stress injury increased the expression of p-p38MAPK, T-p38MAPK, and NF-$\kappa$B, which were significantly decreased by zeaxanthin (*$p<0.05$, **$p<0.01$) (Fig.2e).

**Zeaxanthin alleviates myocardial fibrosis in vitro and in vivo**

Zeaxanthin has been reported to reduce liver fibrosis [9]. However, the role of zeaxanthin in myocardial fibrosis remains unclear. Therefore, we explored the role of zeaxanthin in myocardial fibrosis. First, we used TGF-β1 to induce fibrogenesis in primary CFs. qRT-PCR results showed that TGF-β1 could increase the mRNA levels of FN1, Col-I, and CTGF, compared with the control (Fig.3a). On the other hand, zeaxanthin significantly decreased the mRNA levels of these fibrosis markers ($p<0.05$, **$p<0.01$).
Western blotting further demonstrated the inhibitory effect of zeaxanthin on CFs fibrogenesis induced by TGF-β1 (*p<0.05, **p<0.01) (Fig.3b).

To further explore the function and mechanism of zeaxanthin in myocardial fibrosis, we established an ND group that served as a control and a HFD group where myocardial fibrosis was induced in a C57BL/6 mice background. The myocardial tissue of mice was stained with H&E and Masson's trichrome stain for examining collagen deposition. As shown in Fig.4a, these results proved the successful establishment of the myocardial fibrosis animal model. HFD caused excessive collagen deposition. Zeaxanthin restored the orderly arrangement of cardiomyocytes and reduced collagen deposition, thereby reverting the deleterious effects of HFD. The results of blood lipid determination showed that zeaxanthin could reduce the levels of TG, TC, and LDL in serum (*p<0.05, **p<0.01), which were increased by HFD (Fig.4b). qRT-PCR and western blot results illustrated that zeaxanthin could decrease FN1, Col-I, MMP9, and CTGF expression both at the mRNA and protein levels (*p<0.05, **p<0.01) (Fig.4c, Fig.4d). In addition, HFD activated the expression of TGF-β/Smad2/3 signaling pathway. And after zeaxanthin treatment, the expression levels of TGF-β1 and p-Smad2/3 decreased (*p<0.05, **p<0.01) (Fig.4d).

**Zeaxanthin inhibits myocardial apoptosis in HFD mice**

We further verified the inhibitory effect of zeaxanthin on apoptosis in vivo. Immunofluorescence analysis of ventricular tissue showed that the expression of Bax in the HFD group was significantly increased. Zeaxanthin inhibited the expression of apoptosis markers (Fig.5a). Western blotting further confirmed that zeaxanthin could reduce apoptosis in vivo (*p<0.05, **p<0.01) (Fig.5b).

**Discussion**

Zeaxanthin is an oxygen-containing carotenoid that is commonly found in medicinal plants, vegetables, and fruits [14]. The easy availability and inexpensive nature of zeaxanthin makes it feasible for use alone or in combination with other drugs for the treatment of diseases. Zeaxanthin has many protective effects in the human body, such as reverting the effects of blue light damage to the retina [15], liver fibrosis [8], and synergistic skin antioxidant activity [16]. It also has a protective effect on cardiovascular diseases, including cardiac dysfunction [17], atherosclerosis [18], and coronary heart disease [19]. In the arterial system and blood, zeaxanthin exerts its vascular protective effect in three ways: decreased oxidation of LDL [20], reduced arterial stiffness [21], and prevention of atherosclerosis [22]. However, the specific mechanism and role of zeaxanthin in myocardial injury remains unclear. In this study, we illustrated the protective effect of zeaxanthin on myocardial injury for the first time.

Oxidative stress is accompanied by the excessive production of ROS, leading to myocardial injury and inflammation. Antioxidants can effectively inhibit ROS production. And antioxidants combined with antiinflammatory drugs have a beneficial effect on the treatment of patients with heart failure [23]. Zeaxanthin has strong antioxidant activity and plays an important role in protecting cell membranes and lipoproteins from oxidative stress induced by ROS [24]. In addition, it has been reported that oxidative
stress accompanied by cardiomyocyte apoptosis hinders the recovery of cardiac function after myocardial ischemia-reperfusion injury, which is reversed by inhibition of the ROS-related p38MAPK/NF-κB pathway [25]. Our results showed that zeaxanthin inhibited cardiomyocyte apoptosis, decreased the excessive production of ROS, and reduced the expression of p38MAPK/NF-κB pathway. Thus, we consider that zeaxanthin may inhibit ROS production and reduce cardiomyocyte apoptosis by affecting the expression of p38MAPK/NF-κB signaling pathway (Fig. 2).

Over the last decades, increasing studies described the direct effect of HFD and its related metabolites such as Triglycerides and Free Fat Acids (FFAs), on cardiovascular disease [26]. The HFD and its related metabolites can cause the chronic accumulation of excess fat in the myocardium. It will further lead to various metabolic changes in myocardial cells, activate systems modulating oxidative stress and inflammation, and induce myocardial injury [27]. And HFD is known to promote collagen deposition and fibrosis in heart tissue [28]. These processes are collectively termed “Myocardial lipotoxicity” [29]. Here, we confirmed that HFD could induce myocardial fibrosis and cardiomyocyte apoptosis in the heart tissue of C57BL/6 mice, and the treatment of zeaxanthin could alleviate these pathological changes (Fig. 4, Fig. 5).

TGF-β/Smad2/3 signaling pathway is a key regulator of myocardial fibrosis [30]. The heart tissue is rich in interstitial and perivascular fibroblasts. TGF-β1 can regulate the phenotype and function of CFs by promoting the phosphorylation of intracellular Smad2/3, then stimulate fibroblast activation, induce ECM protein synthesis, promote fibrosis, and diastolic dysfunction [31]. In our study, through experiments in vitro and in vivo, the results showed that zeaxanthin could inhibit the activation of the TGF-β/Smad2/3 signaling pathway. And it also reduced the ECM protein synthesis and myocardial fibrosis at the same time (Fig. 3, Fig. 4). Thus, we consider that the inhibitory effect of zeaxanthin on myocardial fibrosis may be closely related to its influence on TGF-β/Smad2/3 signaling pathway.

In conclusion, our study has confirmed the protective effect of zeaxanthin on myocardial injury. First, we found that zeaxanthin could inhibit cardiomyocyte apoptosis and the production of ROS induced in H9C2 cells by H2O2. And we also found that this protective effect of zeaxanthin was closely related to the inhibition of ROS-related p38MAPK/NF-κB signaling pathway. Next, we used TGF-β1 to induce myocardial fibrosis in CFs. Zeaxanthin was again found to significantly reduce the expression of fibrosis-related markers. Furthermore, we established an animal model of myocardial injury induced by HFD to verify the effects of zeaxanthin on myocardial fibrosis and apoptosis in vivo. In vivo experiments showed that zeaxanthin could reduce apoptosis in heart tissue. In addition, zeaxanthin also could significantly reduce the excessive deposition of collagen in myocardial tissue and reduce the protein expression levels of TGF-β1 and p-Smad2/3. These results show that zeaxanthin has a protective effect against myocardial fibrosis and this protective effect may be closely related to the TGF-β/Smad2/3 signaling pathway. Cardiovascular disease remains a major global health problem, and further research is needed to develop effective treatments and drugs. In this context, the present work assumes immense clinical significance by providing insights into the protective mechanism of zeaxanthin against myocardial injury.
Conclusion

In our study, we have found that zeaxanthin may alleviate HFD and H₂O₂-induced heart injury by regulating TGF-β/Smad2/3 and p38MAPK/NF-κB signaling pathways. Thus, based on our research, we consider that zeaxanthin may play an important role in cardiovascular diseases. It offers a potential treatment for myocardial injury.

Declarations

Ethics approval

All methods including animal experimental procedures were adopted in the study were performed in accordance with ARRIVE guidelines. Animal experimental protocols used in the present study were approved by the Harbin Medical University Research Ethics Committee (2021-SCILLSC-87). The further methods and procedures adopted in the study are in agreement with appropriate guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

Corresponding author will provide the data used in the present work upon request.

Declaration of competing interest

All authors declare that they have no competing interests.

Funding

the National Natural Science Foundation of China (grant no. 81570358).

Author Contributions

This study was designed by Xiang Song, Lichun Qi, and Mingyang Li. The experiments and manuscript preparation were finished by Mingyang Li, Ziguang Song, and Xiao Zhong. The data acquisition and analysis were conducted by Xin Wang and Yanhui Gao. The manuscript was reviewed by Xueqi Li. All authors have read and agreed to publish this manuscript.

Acknowledgements

Not applicable.

References
1. Tallquist MD. Cardiac Fibroblast Diversity. Annu Rev Physiol. 2020; 82:63-78. DOI 10.1146/annurev-physiol-021119-034527.

2. Fan X, Yao Y, Zhang Y. Calreticulin promotes proliferation and extracellular matrix expression through Notch pathway in cardiac fibroblasts. Adv Clin Exp Med. 2018;27(7):887-92. DOI 10.17219/acem/74430.

3. Kumar S, Wang G, Zheng N, Cheng W, Ouyang K, Lin H. HIMF (Hypoxia-Induced Mitogenic Factor)-IL (Interleukin)-6 Signaling Mediates Cardiomyocyte-Fibroblast Crosstalk to Promote Cardiac Hypertrophy and Fibrosis. Hypertension. 2019;73(5):1058-70. DOI 10.1161/HYPERTENSIONAHA.118.12267.

4. Gyongyosi M, Winkler J, Ramos I, Do QT, Firat H, McDonald K. Myocardial fibrosis: biomedical research from bench to bedside. Eur J Heart Fail. 2017;19(2):177-91. DOI 10.1002/ejhf.696.

5. Kang LL, Zhang DM, Jiao RQ, Pan SM, Zhao XJ, Zheng YJ. Pterostilbene Attenuates Fructose-Induced Myocardial Fibrosis by Inhibiting ROS-Driven Pitx2c/miR-15b Pathway. Oxid Med Cell Longev. 2019;2019:1243215.DOI 10.1155/2019/1243215.

6. Wang LP, Fan SJ, Li SM, Wang XJ, Gao JL, Yang XH. Oxidative stress promotes myocardial fibrosis by upregulating KCa3.1 channel expression in AGT-REN double transgenic hypertensive mice. Pflugers Arch. 2017;469(9): 1061-71.DOI 10.1007/s00424-017-1984-0.

7. Firdous AP, Sindhu ER, Kuttan R. Hepato-protective potential of carotenoid meso-zeaxanthin against paracetamol, CCl4 and ethanol induced toxicity. Indian J Exp Biol. 2011;49(1):44-9.

8. Chamberlain SM, Hall JD, Patel J, Lee JR, Marcus DM, Sridhar S. Protective effects of the carotenoid zeaxanthin in experimental nonalcoholic steatohepatitis. Dig Dis Sci. 2009;54(7):1460-4. DOI 10.1007/s10620-009-0824-2.

9. Firdous AP, Kuttan R. Chemo protective activity of carotenoid meso-zeaxanthin against doxorubicin-induced cardio toxicity. J Exp Ther Oncol. 2012;10(2):101-6.

10. Polidori MC, Savino K, Alunni G, Freddio M, Senin U, Sies H. Plasma lipophilic antioxidants and malondialdehyde in congestive heart failure patients: relationship to disease severity. Free Radic Biol Med. 2002;32(2):148-52. DOI 10.1016/S0891-5849(01)00782-1.

11. Adluri RS, Thirunavukkarasu M, Zhan L, Maulik N, Svennevig K, Bagchi M. Cardioprotective efficacy of a novel antioxidant mix VitaePro against ex vivo myocardial ischemia-reperfusion injury. Cell Biochem Biophys. 2013;67(2):281-6. DOI 10.1007/s12013-011-9300-7.

12. Tavani A, Gallus S, Negri E, Parpinel M, La Vecchia C. Dietary intake of carotenoids and retinol and the risk of acute myocardial infarction in Italy. Free Radic Res. 2006;40(6):659-64. DOI 10.1080/10715760600615649.

13. Dwyer JH, Paul-Labrador MJ, Fan J, Shircore AM, Merz CN, Dwyer KM. Progression of carotid intima-media thickness and plasma antioxidants: the Los Angeles Atherosclerosis Study. Arterioscler Thromb Vasc Biol. 2004;24(2):313-9. DOI 10.1161/01.ATV.0000109955.80818.8a.

14. Ma L, Lin XM. Effects of lutein and zeaxanthin on aspects of eye health. J Sci Food Agric. 2010;90(1):2-12. DOI 10.1002/jsfa.3785.
15. Gheorghe A, Mahdi L, Musat O. Age-Related Macular Degeneration. Rom J Ophthalmol. 2015;59(2):74-7.

16. Roberts RL, Green J, Lewis B. Lutein and zeaxanthin in eye and skin health. Clin Dermatol. 2009;27(2):195-201. DOI 10.1016/j.clindermatol.2008.01.011.

17. El-Baz FK, Hussein RA, Saleh DO, Abdel Jaleel GAR. Zeaxanthin Isolated from Dunaliella salina Microalgae Ameliorates Age Associated Cardiac Dysfunction in Rats through Stimulation of Retinoid Receptors. Mar Drugs. 2019;17(5). DOI 10.3390/md17050290.

18. Ciccone MM, Cortese F, Gesualdo M, Carbonara S, Zito A, Ricci G. Dietary intake of carotenoids and their antioxidant and anti-inflammatory effects in cardiovascular care. Mediators Inflamm. 2013;2013:782137. DOI 10.3390/md17050290.

19. Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE. Fruit and vegetable intake in relation to risk of ischemic stroke. JAMA. 1999;282(13):1233-9.

20. Carpenter KL, van der Veen C, Hird R, Dennis IF, Ding T, Mitchinson MJ. The carotenoids β-carotene, canthaxanthin and zeaxanthin inhibit macrophage-mediated LDL oxidation. FEBS Lett. 1997;401(2-3):262-6. DOI 10.1016/S0014-5793(96)01488-3.

21. Dwyer JH, Navab M, Dwyer KM, Hassan K, Sun P, Shircore A. Oxygenated carotenoid lutein and progression of early atherosclerosis: the Los Angeles atherosclerosis study. Circulation. 2001;103(24):2922-7. DOI 10.1161/01.CIR.103.24.2922.

22. Zou Z, Xu X, Huang Y, Xiao X, Ma L, Sun T. High serum level of lutein may be protective against early atherosclerosis: the Beijing atherosclerosis study. Atherosclerosis. 2011;219(2):789-93. DOI 10.1016/j.atherosclerosis.2011.08.006.

23. Aimo A, Castiglione V, Borrelli C, Saccaro LF, Franzini M, Masi S. Oxidative stress and inflammation in the evolution of heart failure: From pathophysiology to therapeutic strategies. Eur J Prev Cardiol. 2020;27(5):494-510. DOI 10.1177/2047487319870344.

24. Sies H, Stahl W. Vitamins E and C, beta-carotene, and other carotenoids as antioxidants. Am J Clin Nutr. 1995;62(6 Suppl):1315S-21S. DOI 10.1093/ajcn/62.6.1315S.

25. Guo W, Liu X, Li J, Shen Y, Zhou Z, Wang M. Prdx1 alleviates cardiomyocyte apoptosis through ROS-activated MAPK pathway during myocardial ischemia/reperfusion injury. Int J Biol Macromol. 2018;112:608-15. DOI 10.1016/j.ijbiomac.2018.02.009.

26. Manrique C, DeMarco VG, Aroor AR, Mugerfeld I, Garro M, Habibi J. Obesity and insulin resistance induce early development of diastolic dysfunction in young female mice fed a western diet. Endocrinology 2013; 154:3632–42.

27. Ndisang JF, Vannacci A, Rastogi S. Oxidative stress and inflammation in obesity, diabetes, hypertension, and related cardiometabolic complications. Oxid Med Cell Longev 2014; 2014:506948.

28. Jiang J, Li Y, Liang S, Sun B, Shi Y, Xu Q. Combined exposure of fine particulate matter and high-fat diet aggravate the cardiac fibrosis in C57BL/6J mice. J Hazard Mater. 2020;391:122203. DOI 10.1016/j.jhazmat.2020.122203.
29. Boden G. Obesity, insulin resistance and free fatty acids. Current opinion in endocrinology, diabetes, and, obesity. 2011; 18:13.

30. Hu HH, Chen DQ, Wang YN, Feng YL, Cao G, Vaziri ND. New insights into TGF-beta/Smad signaling in tissue fibrosis. Chem Biol Interact. 2018;292:76-83. DOI 10.1016/j.cbi.2018.07.008.

31. Russo I, Cavalera M, Huang S, Su Y, Hanna A, Chen B. Protective Effects of Activated Myofibroblasts in the Pressure-Overloaded Myocardium Are Mediated Through Smad-Dependent Activation of a Matrix-Preserving Program. Circ Res. 2019;124(8):1214-27.

Figures

**Figure 1**

Effect of zeaxanthin on the activity of H9C2 cells (a) CCK-8 assay was used to detect the optimal time of zeaxanthin treatment in H9C2 cells. The results were measured at 0, 12, 24, and 48 h of zeaxanthin treatment. n = 3. (b) MTT assay was used to detect the effect of zeaxanthin on cell survival rate. n = 6. (c) Western blotting analysis of Bcl2, Bax, and cleaved-caspase-3. GAPDH was taken as an internal control gene. All data were expressed as mean ± SEM. Differences in expression were analyzed by Student’s t-test and one-way ANOVA (n = 3. * p<0.05, ** p<0.01).
Zeaxanthin suppresses cardiomyocyte apoptosis induced by H2O2 (a) TUNEL staining of H9C2 cells and statistical analysis. Magnification, 400x. (b) Detection of cardiomyocyte apoptosis using an Annexin V-FITC/PI apoptosis kit. (c) Western blotting analysis of Bcl2, Bax, and cleaved-caspase-3. GAPDH was taken as an internal control gene. (d) Production of ROS in H9C2 cells detected using a DCFH-DA probe. Magnification, 400x. (e) Western blotting analysis of p-p38MAPK and p-NF-kB. GAPDH was taken as an internal control gene. All data were expressed as mean ± SEM. Differences in expression were analyzed by Student’s t-test and one-way ANOVA (n = 3. * p<0.05, ** p<0.01).
Figure 3

Zeaxanthin reduces CFs activation induced by TGF-β1 (a) qRT-PCR analysis of FN1, Col-I, and CTGF expression. (b) Western blotting analysis of FN1 and Col-I. GAPDH was taken as an internal control gene. All data were expressed as mean ± SEM. Differences in expression were analyzed by Student's t-test and one-way ANOVA (n = 3. * p<0.05, ** p<0.01).
Zeaxanthin reduces myocardial fibrosis induced by HFD (a) H&E and Masson staining were used to detect the histomorphology and degree of fibrosis of mice myocardial tissue. Magnification, 200x. (b) Detection of blood lipids in mice serum. (c) qRT-PCR analysis of fibrosis-related markers expression in myocardial tissue. (d) Western blotting analysis of FN1, Col-I, Smad2/3, p-Smad2/3, and TGF-β1. GAPDH was taken as an internal control gene. All data were expressed as mean ± SEM. Differences in expression were analyzed by Student’s t-test and one-way ANOVA (n = 3. * p<0.05, ** p<0.01).
Figure 5

Zeaxanthin inhibits myocardial apoptosis induced by HFD (a) Immunofluorescence was used to detect Bax expression in myocardial tissue. Magnification, 200x. (b) Western blotting analysis of Bcl2, Bax, and cleaved-caspase-3. GAPDH was taken as an internal control gene. All data were expressed as mean ± SEM. Differences in expression were analyzed by Student's t-test and one-way ANOVA (n = 3. *p<0.05, **p<0.01).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Westernblot.pdf