Conference Paper

The Effect of Astaxanthin on Glutathione Levels in Damaged Liver Tissues of Male Wistar Rats Induced By Oral Formaldehyde

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Abstract

Formaldehyde is an aldehyde derivative which is illegally used as a food preservative. An impaired liver function could result from exposure to formaldehyde through the process of oxidative stress. Astaxanthin is expected to increase the levels of glutathione (GSH), which is a natural antioxidant in the human body. An antioxidant can be used to inhibit formaldehyde-induced free radicals. This study aimed to determine the effect of astaxanthin on GSH levels in damaged liver tissues of male Wistar rats induced by oral formaldehyde. This study was an experimental study with a posttest-only control group design. Thirty rats were divided into normal control group; the negative control group which was given only formaldehyde; Group 1, 2, and 3 which was given a 12, 24, and 48 mg/day dose of astaxanthin. GSH levels of each group were measured using the Ellman Method and the data were analyzed statistically using SPSS 23.00. The value of GSH levels in treatment group 1 was 4.492 ± 0.29 µg/ml, treatment group 2 was 6.075 ± 0.96 µg/ml, and treatment group 3 was 5.132 ± 0.52 µg/ml. GSH levels in group 2 and 3 were significantly different compared with the negative control group (LSD, p < 0.05). However, GSH levels in group 1 were not significantly different compared with the normal control group and negative control group (LSD, p > 0.05). Astaxanthin could increase GSH levels in damaged liver tissues of male Wistar rats induced by oral formaldehyde.

Keywords: astaxanthin, formaldehyde, glutathione

1. Background

The liver is the largest and most important metabolic organ in the human body, with a very complex function for maintaining body homeostasis [1, 2]. Liver damage can be caused by various metabolic, toxic, microbial, and circulatory disturbances. In most cases, the disease process occurs mainly in the liver. The hepatic disease itself is a cause of large burden disease across Europe [3]. World Health Organization (WHO) states that
disease caused by a hepatic impairment is the cause of high mortalities in Indonesia [4]. Liver damage may be caused by free radicals from formaldehyde metabolism. Formaldehyde (CH\(_2\)O) is a derivative of aldehydes, which are highly reactive, flammable and can explode in the air. Formaldehyde is present in many types of building construction materials and is also used in hospitals, research, and laboratories as agents of sterilization, antibacterial, and cadaver preservation [5-7]. However, in Indonesia formaldehyde has been widely abused as a preservative in foods [8]. Based on the data from the Food and Drug Supervisory Agency (BPOM), there are still many food traders using formaldehyde as a preservative in various regions of Indonesia from 2011 to 2014. The Indonesian government has established some laws that formaldehyde use is prohibited for food preservative (References). Law No. 7/1996 about Food, Law No. 8/1999 about Consumer Protection and Government Regulation No. 28 of 2004 about Food Safety. In West Kalimantan, a variety of foods such as tofu, yellow noodles, sweets, fish bloated, red cherries, and corn vermicelli contain formaldehyde, which is found in some traditional markets [9, 10].

Formaldehyde exists in our body with a normal level of approximately 2.6 mg / L in the blood [11]. Excess formaldehyde will bind into natural antioxidant compounds in the body; for example, reduced glutathione (GSH), and will conjugate to form S-hydroxymethyl glutathione, causing a drastic reduction in the amount of GSH [12]. Formaldehyde may also increase the production of Reactive Oxygen Species (ROS) compounds in the body, causing oxidative stress. Oxidative stress is an unbalanced state between the antioxidants and the prooxidant present in the body that can further lead to the hepatic cell damage and death [13]. Under conditions of oxidative stress, there is a decrease in GSH levels in the body [14]. GSH is an endogenous antioxidant that has several functions in tissue protection from oxidative damage and maintains the stability of the intracellular environment. GSH prevents free radical formation by functioning as a substrate in the decomposition reaction of a non-radical compound H\(_2\)O\(_2\) into H\(_2\)O and O\(_2\) molecules catalyzed by glutathione peroxidase (GPx) enzyme. GSH itself will turn into oxidized glutathione (GSSG) in this reaction [15].

The use of antioxidants has begun to develop recently as the understanding of its role in inhibiting degenerative diseases caused by the accumulation of ROS, such as heart disease, atherosclerosis, cancer, and the symptoms of aging [16]. Antioxidants are chemical compounds that can contribute one or more electrons to free radicals so that free radicals can be inhibited [17]. The human body doesn't have excessive amounts of antioxidants, so if there is excessive radical exposure, then the body needs exogenous
antioxidants [18]. One of the known exogenous antioxidants that have very strong effects is astaxanthin [19].

Astaxanthin is a carotenoid pigment that naturally found in foods such as shrimp, crabs, lobsters, freshwater fish, marine fish, salmon, as well as several types of bacteria and fungi [20, 21]. *Haemococcus pluvialis*, one species of green algae, is the largest natural source of astaxanthin [22]. The antioxidant capability of astaxanthin is primarily caused by its unique chemical structure, allowing astaxanthin to capture a single ROS. Biswal reported that showed that the effect of astaxanthin as an antioxidant is 10 times stronger than other carotenoids such as lutein, zeaxanthin and ß-carotene and 100 times stronger than α-tocopherol [19]. Chan et al reported astaxanthin supplementation significantly improved the production of depleted GSH and reduced ROS production [23]. Otton et al reported that endogenous antioxidants such as GSH, GPx, and Superoxide Dismutase (SOD) will increase after 45 days of astaxanthin administration [24]. Based on the background above, the aim of this study was to investigate the effect of astaxanthin on glutathione levels in damaged liver tissues of male Wistar rats induced by oral formaldehyde.

2. Materials and Methods

This was an experimental research study with the post-test only control group design. Pure astaxanthin powder derived from *Haemococcus pluvialis* extract was purchased from Futamed Industries. The sample used was liver tissues of Wistar rats. A total of 30 rats, which aged two to three months, were divided into 5 groups: control (normal and negative) groups and treatment groups (group 1, 2 and 3). Rats were adapted for 14 days and treatment was administered for the next 28 days. The normal control group is given only standard feed and drink. The negative control group was given oral formaldehyde induction for 14 days. Treatment group 1, 2 and 3 were given oral formaldehyde induction for 14 days, followed by administration of astaxanthin dose 12, 24 and 48 mg/day.

GSH level assay of hepatic tissue was tested by using Ellman Method Measurements [25] and was performed after 4 weeks of treatment. The study period was from September to November 2015 at the research laboratory in Tanjungpura University. All data are reported as mean ± SD. Statistical tests were performed using One-way Anova test, followed by Post Hoc Test. A p value of <0.05 was considered to be statistically significant.
3. Results

From the research that has been done, the mean value of GSH levels of hepatic tissue can be seen in Figure 1.

![Figure 1: Effect of Astaxanthin on Hepatic GSH Levels. Each value represents mean value ± SD. * p > 0.05 by One-way Anova test followed by Post Hoc test; ** p < 0.05. C(0), normal control; C(-), negative control; T(1), treatment of astaxanthin dose 12 mg/day; T(2), treatment of astaxanthin dose 24 mg/day; T(3), treatment of astaxanthin dose 48 mg/day.]

We have demonstrated the effect of astaxanthin on hepatic GSH levels in rat liver damage induced by oral formaldehyde. Figure 1 showed that the lowest mean value of GSH levels was in the negative control group and the highest was in the treatment group 2. Furthermore, GSH levels in the treatment group 1 were not significantly different from the normal control group. However, there was a significant improvement of GSH levels in the treatment group 2 compared with the normal control group and the negative control group. In the treatment group 3, there was a significant difference with the negative control group but was not different significantly with the treatment group 3.

4. Discussion

We conducted in vivo studies to investigate the effects of astaxanthin, a natural antioxidant, on hepatic GSH levels. We demonstrated that post-treatment with astaxanthin could improve hepatic GSH levels. The three treatment groups tested had higher GSH levels when compared with the negative control group, although the treatment group 1
was not statistically significant compared to the negative control group. A previous study by Kang et al (2001) [26] and Wang et al (2014) [27] also indicated that administration of astaxanthin could increase GSH levels of tissue, in hepatic and renal tissue. These results collaborated those of previous studies.

The effect of elevated GSH levels on hepatic tissue by astaxanthin may be caused by several mechanisms, such as increased GSH biosynthesis through activation of the NRF2 pathway and decreased ROS through its work as a scavenger, thus assisting GSH in eliminating ROS and inhibiting lipid peroxidation in hepatic cells due to exposure to formaldehyde [28, 29]. Astaxanthin has the ability to trigger NRF2 to mediate endogenous antioxidant systems in the body, by increasing the expression of various antioxidant enzymes that play a role in biosynthesis and GSH function. It should be noted that there are two GSH biosynthesis pathways, among others through the de novo synthesis pathway and the resynthesis pathway. GSH biosynthesis via the de novo pathway takes place by utilizing the amino acids available in the body as its constituent materials, including glutamate, cysteine, and glycine, already present in the cell or transported from the extracellular. The resynthesis pathway occurs by reducing GSSG to GSH with the help of Glutathione Reductase (GR) enzyme and requires NADPH [30].

In addition to activating the NRF2 system, astaxanthin as an antioxidant scavenger could be effective in counteracting various ROS in the cells. Astaxanthin has a wide antioxidant capacity, due to its unique chemical structure, distinct from other antioxidants. It has polar and nonpolar clusters, so it can enter all parts of the cell membrane in a linear fashion. These structures and positions cause astaxanthin to counteract ROS both outside and inside the cell [31, 32].

The results showed that the treatment group 2 had higher levels of GSH than the treatment group 3. In other words, although the dose of treatment group 3 was greater than the treatment group 2, the effect on GSH was lower instead. In the body, there is a mechanism for setting up homeostasis for GSH levels in various tissues, one of which is liver. GSH is an important and required tripeptide by all cells, with normal levels of about 1-10mM in all cell types, including hepatocyte cells. GSH deficiency can cause oxidative stress, which causes aging, as well as various diseases in humans that include neurodegenerative diseases, liver disease, diabetes, cystic fibrosis, AIDS, heart disease and cancer. However, when intracellular GSH levels meet the needs of the cell, both in the prevention of free radicals and other functions, there will be a GSH degradation mechanism, using the y-Glutamyl-transpeptidase (γ-GT) enzyme that will break GSH into cysteine glycine and glutamate residues which will be transported to other amino acid acceptors. Furthermore, cysteine glycine will be broken down by dipeptidase enzyme
to produce amino acids cysteine and glycine which are then allocated for other needs [33].

The effective dose in this study was treatment group 2, with a dose of 24 mg/day. This was due to a statistically significant increase in GSH levels when compared with the normal control group and negative control group.

Although this research has reached its aims, there was an unavoidable limitation. For animal research outcome, there were some intrinsic and extrinsic factors that could affect the result, such as genetics, nutritional and immune status, endocrine factors, cage design, temperature, humidity, ventilation, noise, etc. In addition, due to a limited fund, this research was conducted to see the effect of astaxanthin on hepatic GSH levels only. Therefore, further research are needed to see the effect of astaxanthin on another organ, different biomolecular indicator or method.

5. Conclusion

Astaxanthin could increase GSH levels in damaged liver tissues of male Wistar rats induced by oral formaldehyde

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Original Article
Protective effects of astaxanthin against diabetic retinal vessels and pro-inflammatory cytokine synthesis

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Abstract: Background: Diabetic retinopathy (DR) is a common microvascular complication of diabetes and a leading cause of blindness. Astaxanthin (AST) is a naturally occurring carotenoid with many biological protective activities. The purpose of the present study was to investigate the protective effects of AST on DR in a rat model of type 1 diabetes mellitus (DM) and to examine the mechanisms involved. Methods: An intraperitoneal injection of 1% streptozotocin was used to prepare the rat model of diabetes. Rats were randomly assigned to one of three groups: untreated control, diabetes + olive oil (DM), or DM+AST (n = 8 per group). The AST group received 20 mg/kg/day AST dissolved in olive oil by gavage. The DM group received an equal volume of olive oil. During the study, blood glucose levels and body weights were measured every two weeks. After six months, retinas were excised to prepare the retinal capillary network. Endothelial cell to pericyte ratio (E/P) and the numbers of acellular capillary strands were compared among different experimental groups. Formation of advanced glycation end products (AGEs) and expression of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and caspase-3 in retinal tissues were assessed by immunohistochemistry and RT-PCR. Results: AST slightly increased body weight but had no significant effects on blood glucose levels. E/P and numbers of acellular strands in the DM+AST group were lower than those in the DM group. Expression of AGE, IL-6, TNF-α, and caspase-3 in retinal tissues decreased compared with those of the DM group. All differences between the groups were statistically significant (P < 0.05). Conclusion: AST can protect pericytes from apoptosis and delay development and progression of DR in streptozotocin-induced diabetic rats. Additionally, it can reduce generation of AGEs, release of inflammatory cytokines (IL-6, TNF-α), and cleavage of caspase-3, which may mediate pericyte apoptosis.

Keywords: Astaxanthin, diabetic retinopathy, anti-apoptosis, anti-inflammation

Introduction

Diabetes is a systemic metabolic disease characterized by chronic hyperglycaemia. The retina is one of the most vulnerable tissues [1-3]. Diabetic retinopathy (DR) is the most common microvascular complication of diabetes, known to be the leading cause of blindness [4]. DR is characterized by progressive retinal vasculopathy, leading to breakdown of the blood-retina barrier (BRB), leakage of retinal vessels, oedema, ischaemia, and neovascularization [5]. Mechanisms of the development of DR are not fully understood, but some distinct biochemical pathways have been associated with development of DR, including pericyte apoptosis, advanced glycation end-product (AGE) formation, oxidative stress, and inflammation [6]. Therefore, researchers have focused on methods to delay progression of DR by inhibiting pericyte apoptosis and reducing accumulation of AGEs and inflammatory response [7, 8].

Astaxanthin (3,3’-dihydroxy-β,β’-carotene-4,4’-dione, AST) is a naturally occurring carotenoid reported to have a wide variety of biological functions, including anti-inflammatory, anti-apoptosis, antioxidant (10 times higher than that of other carotenoids), anti-cancer, and neuroprotective effects [9-11]. The study of AST has received increasing attention. AST has been reported to have the capacity to reduce inflammation and apoptosis [12]. In a study conducted by Dong et al., treating cultured RGC-5 ganglion cells with astaxanthin decreased hydrogen peroxide-induced apoptosis in the control group. However, molecular mechanisms by which AST inhibits apoptosis of peri-
cytes in streptozotocin (STZ)-induced diabetic rats remain unclear [13].

The present study evaluated the protective effects of AST in a rat model of type 1 diabetes mellitus (DM) by determining its effects on general symptoms of DR, pericytes, inflammatory response in the retina, and retinal vessel damage.

**Materials and methods**

**Animals**

Twenty-four male Sprague-Dawley rats, aged 6 weeks and weighing 260-280 g, were purchased from Beijing HFK Bioscience Co. Ltd. (SCXK (Jing) 2014-0005). The animals were kept in an ordinary housing facility in keeping with national standards (Laboratory Animal Requirements of Environment and Housing Facilities [GB 14925-2001]). All experimental procedures were performed according to the ARVO statement for Use of Animals in Ophthalmic and Vision Research.

Animals were fed and housed in the center and maintained under conditions of controlled temperature (20-24°C), relative humidity of 40%, and a 12-hour light-dark cycle. All rats had free access to food and water throughout the study. Bedding was changed daily and the rats were monitored regularly.

**Experimental procedure**

The animals were fasted for 12 hours prior to establishing the diabetic model. Rats in the DM groups were injected intraperitoneally with 1% STZ (Sigma-Aldrich, St. Louis, MO, USA) and dissolved in citrate buffer (pH 4.5) at 60 mg/kg. Rats in the negative control group received the same volume of citrate buffer only. A blood glucose level > 16.7 mmol/L for three consecutive days indicated successful establishment of the DM model. Treatment with AST (Sigma-Aldrich, St. Louis, MO, USA) was started 3 days after diabetes confirmation and continued for 24 weeks. Dosage (in mg/kg) was calculated with the formula given below: Dosage of AST = Minimum Inhibitory Concentration × 20 [14].

The animals were allocated randomly to three groups of eight animals each: [Control group], C: normal rats received 0.9% NaCl by daily gavage and served as a negative control group. [Diabetic group], DM: diabetic rats received olive oil by gavage each morning. [Diabetic + AST-treated group], DM+AST: diabetic rats received AST (20 mg/kg/day) in olive oil by gavage each morning.

During the experimental period, all rats had free access to food and water. Blood glucose levels and body weight were measured every 2 weeks. At the end of the study, the rats were euthanized with an overdose of chloral hydrate anaesthesia. Eye globes were immediately removed, weighed, marked, and stored at -80°C until analysis.

**Retinal capillary network preparation**

Eye globes were placed in 4% paraformaldehyde for 72 hours. The cornea and lens were then removed under a microscope. The retina was separated from the choroid carefully using a blunt metal spatula. The optic nerve was removed with scissors, carefully, to not damage the retina. The intact retina was divided into four pieces and they were then put in a test tube with trypsin solution (3% in sodium phosphate buffer, pH 7.2-7.4). The retina was digested for 3 hours in a water bath at 37°C, mixing 3-4 times during the digestion. The inner limiting membrane was peeled away and a cat’s whisker was used to clear the remaining nerve tissue on the retinal vascular network under a microscope. Tissue pieces were placed on a slide and allowed to air-dry. After periodic acid-Schiff (PAS) staining, retinal capillary cells (pericytes) were counted across five views of the central area (400 × magnification) under a microscope. Tissue pieces were placed on a slide and allowed to air-dry. After periodic acid-Schiff (PAS) staining, retinal capillary cells (pericytes) were counted across five views of the central area (400 × magnification) under a microscope. Tissue pieces were placed on a slide and allowed to air-dry. After periodic acid-Schiff (PAS) staining, retinal capillary cells (pericytes) were counted across five views of the central area (400 × magnification) under a microscope. Endothelial/pericyte (E/P) ratio was calculated and compared between the different experimental groups. Numbers of acellular collapsed capillary strands were also counted in five views (400 × magnification) per retina.

**Immunohistochemical staining**

Eyeballs were fixed in 4% paraformaldehyde for 3 days (Sigma-Aldrich, St. Louis, MO, USA), placed vertically in tissue cassettes, and paraffin-embedded. After deparaffinization and rehydration, sections were stained with haematoxylin and eosin for examination of pathological changes in morphology. To visualise cytokines, sections were first incubated with 0.1% hydrogen peroxide in phosphate-buffered saline (PBS) for 30 minutes to block the activity of
endogenous peroxidases. After washing three times with PBS, sections were blocked with 10% goat serum at 37°C for 30 minutes and then incubated with rabbit anti-rat AGE (1:100), IL-6 (1:100), TNF-α (1:100), and cleaved caspase-3 (1:100) primary antibodies (Sigma-Aldrich, St. Louis, MO, USA) at 4°C for 24 hours. After washing, sections were then incubated with species-appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma) for 30 minutes at 37°C. Slides were washed three times with PBS and stained with 3,3’-diaminobenzidine (DAB, Zhongshan Golden Bridge Biotechnology, Beijing, China). A positive signal in the retina was brown. Five visual fields of each section were selected randomly (400 × magnification), captured, and integrated optical density (IOD) was calculated using Image-Pr. Plus 6 image analysis.

**Real-time PCR**

Left posterior segments were put in a Petri dish filled with distilled water. The retina was removed from the pigment epithelium under a dissecting microscope for preparation of total RNA. Total RNA was extracted from the retinas with TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA). Reverse-transcriptase PCR was used to measure mRNA levels of RAGE, IL-6, TNF-α, and caspase-3 in retinal tissue. Concentration and purity of total RNA was measured using an ultraviolet spectrophotometer (Takara, Shiga). Oligonucleotides were used as primers in a 25-µL reaction system (Takara). Oligo sequences are shown in Table 1. Moloney murine leukaemia virus reverse transcriptase (M = MLV RT) was used to synthesize cDNA (Takara). RT-PCR Master Mix (GoTaq®, Promega, Madison, WI, USA) was used for amplification and a LightCycler® 480 system (Roche Diagnostics Corporation, Indianapolis, IN, USA) was used for detection. After completion of the reaction, the threshold cycle value (Ct) for all curves was calculated. Using a base of two, the difference in exponents of the values obtained was used to calculate relative expression of the target genes. Each experiment was performed with three or more replicates. The melting curve of PCR products showed a single peak. Agarose gel electrophoresis was used for further verification of product size.

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Student’s t-tests and one-way analysis of variance (ANOVA) with Tukey’s multiple comparison testing were used in Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) to compare differences between groups. Differences with \( P < 0.05 \) are considered statistically significant.

**Results**

**Body weight and blood glucose**

Body weight and blood glucose measurements are shown in Figure 1. Initial body weights were similar in all groups, but final body weights were lower in the diabetic rats than in control rats. Average body weight in the DM+AST group was higher than that of the DM group at all time points (Figure 1A). As expected, injection of STZ increased blood glucose levels after 24 weeks, but there were no statistically significant differences in blood glucose levels between the DM groups with or without AST (Figure 1B).

**PAS staining of retinal vessel preparations**

Results of retinal vessel staining are shown in Figure 2. Retinal capillary networks are visible at a high magnification. In the C group, the main artery was round, uniform, and strongly stained. The vein was lightly stained and had a large diameter. Capillaries were reasonably straight, of uniform diameter, and interconnected into a network (Figure 2A, 2D). At a higher magnification, the capillaries were mainly composed of two types of cells. One cell type was endothelial cells, which had a large, oval, or round nucleus that was stained lightly and was generally locat-
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ed in the central part of the capillary (Figure 2B, black arrow). The other cell type was the pericyte, which had a small, round, or triangular nucleus that was stained deeply and was generally located at one side of the capillary wall (Figure 2A, black arrow). In the DM group, the retinal arteriovenous trunk and branches appeared tortuous at a low magnification, with capillary network disorder (Figure 2E). At a higher magnification, the capillaries had expanded. There were clear indications of local steno-sis, kinks in the capillary loops, dense microvascular networks (Figure 2B), proliferation of endothelial cells, and pericyte ghosts. The morphology of capillaries in the DM+AST group was between that of the control and DM groups, with reduced vascular tortuosity, dilatation, and stenosis (Figure 2C, 2F).

E/P and acellular capillaries

As shown in Figure 3, the E/P ratio was higher in DM and DM+AST groups than in the C group. Compared with that of the DM group, E/P ratio was lower in the DM+AST group. This difference was statistically significant. Formation of acel-lular strands was more frequent in diabetic rats than in control rats. Data showed that the number of acellular strands in the DM group was higher than the DM+AST group.

Histological examination and immunohistochemistry of AGEs, inflammatory proteins, and caspase-3 in retinas

Under low magnification, the structure of each layer of the retina in the C group was clearly visualized (Figure 4A). Inner and outer nuclear layers were clearly visualized. In the DM group (Figure 4B), the density of the nuclear layers was reduced, cell arrangement was disorganized, and tissue contained void spaces. In the DM+AST group (Figure 4C), the structures of the retina remained intact and the density of the nuclear layers was higher than that of the DM group. This study examined expression of AGEs, IL-6, TNF-α, and cleaved caspase-3 by immunohistochemistry of the retinas (Figures 5 and 6). Positively stained cells were quantified in images. IOD showed higher expression levels of AGEs, IL-6, TNF-α, and cleaved caspase-3 in the retinas of the DM group, compared to those of C and DM+AST groups (P < 0.05).

Effects of AST on mRNA levels of RAGE, inflammatory proteins, and caspase-3 in retinas

The present study observed that there were statistically significant differences between groups in all measured mRNA levels. As shown in Figure 7, relative mRNA expression of RAGE, IL-6, TNF-α, and caspase-3 in retinas was quantified. According to results, AGEs, IL-6, TNF-α, and caspase-3 mRNAs were significantly elevated in retinas of the DM group, compared to those of C and DM+AST groups (P < 0.05).

Discussion

Although AST is not naturally present in the human retina, it easily crosses the BRB, subsequently protecting retinal ganglions [15]. However, AST has never been reported to protect pericytes to maintain normal vascular and reduce inflammatory response. For these rea-sons, it was hypothesized that AST could inhibit pericyte apoptosis by decreasing levels of pro-inflammatory cytokines and pro-apoptotic factors in the retina.

Previous reports have documented that AST can improve diabetic symptoms and delay progression of diabetic complications in experimental diabetes models. These improvements include reducing blood sugar levels, attenuat-
Astaxanthin against diabetic retinal vessels and inflammatory
ing DR, and preventing diabetic nephropathy [12, 13]. Similarly, it was found that AST could partially ameliorate the loss of body weight in diabetic rats. However, present data showed that blood glucose levels in diabetic rats were not affected by AST treatment, similar to the data of Chan et al. [16].

Retinal capillaries contain endothelial cells, astrocytes, and pericytes. In the inner BRB, endothelial cells are surrounded by pericytes and the foot processes of astrocytes [17]. The main function of pericytes is to maintain vascular stability [18]. Apoptosis of pericytes leads to formation of pericyte ghosts, which increase the numbers of acellular capillaries and proliferation of endothelial cells [19]. Apoptotic cell death alters the retinal structures and stimulates release of inflammatory mediators [20]. Inner BRB damage results in leakage of retinal vessels, basement membrane thickening, endothelial cell damage, macu-
Astaxanthin against diabetic retinal vessels and inflammatory oedema, and neovascularization [6]. Accumulating evidence has suggested that pericyte apoptosis occurs at an early stage and is a hallmark of DR [21]. However, underlying molecular mechanisms have not been clearly characterized. The present study observed that rats with diabetes showed higher E/P ratios and greater numbers of acellular capillaries compared to control rats. Data showed that 20 mg/kg AST can decrease the ratio of E/P and numbers of acellular capillaries, compared to those of the DM group. However, ghost cells

Figure 4. Haematoxylin and eosin staining of representative sections of rat retina. A. Group C; B. group DM; C. Group DM+AST. All images are 400 × magnification. Abbreviations: C, control rats; DM, diabetic rats; DM+AST, diabetic rats receiving astaxanthin.

Figure 5. Immunohistochemistry of retinal sections (400 × magnification). Positive expression is indicated by dark brown staining. Abbreviations: C, control rats; DM, diabetic rats; DM+AST, diabetic rats receiving astaxanthin; AGE, advanced glycation end products; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α.
Astaxanthin against diabetic retinal vessels and inflammatory

Figure 6. Quantification of immunohistochemistry data from Figure 5. (A) AGE levels, (B) IL-6 levels, (C) TNF-α levels, (D) Cleaved caspase-3 levels. Expression levels were quantified from immunohistochemistry images, as described in the Methods section. Plotted values are mean ± standard deviations (n = 8). *P < 0.05 for the indicated comparison. Abbreviations: C, control rats; DM, diabetic rats; DM+AST, diabetic rats receiving astaxanthin; AGE, advanced glycation end products; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; IOD, integrated optical density.

Figure 7. Real time reverse-transcriptase PCR of mRNA in the three different treatment groups. (A) Levels of RAGE mRNA, (B) Levels of IL-6 mRNA, (C) Levels of caspase-3 mRNA. All values were normalized to that of GAPDH mRNA. Plotted are the means ± standard deviations (n = 8). P < 0.05, **P < 0.01 and ***P < 0.001 for indicated comparisons.

AGES are the adducts of sugar aldehyde groups and amino nucleophiles in proteins that form an Amadori product in a process termed protein glycation [23]. A previous study reported that AGES play a role in DR by inducing apoptosis and inflammation in retinal pericytes via interaction with a receptor for AGE (RAGE) [24]. This process can upregulate the pro-apoptotic gene, Bax, to promote development of apoptosis in bovine retinal capillary pericytes [25]. Interaction of AGES and RAGE activates nuclear factor-kB (NF-kB) and generates pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α [26]. The present study demonstrated increased levels of AGES in diabetic rats, suggesting that they are responsible for retinal pericyte apoptosis. This result was similar to results by Kim et al. [27]. Anti-apoptotic effects of AST are probably due and acellular capillaries were present in both DM and DM+AST groups. In another study, Robison et al. reported that the number of pericyte ghosts increased after eight months of diabetes [22]. Thus, a limitation of the present study was that it is difficult to separate pericytes from endothelial cells. Cell ratio changes observed in the capillaries of diabetic rats could be the result of changes in endothelial cells, pericytes, or both.
Astaxanthin against diabetic retinal vessels and inflammatory to its inhibitory effects on production of AGEs in the retina.

Chronic inflammation plays a key role in progression of DR and exacerbates DR deterioration [8]. Pro-inflammatory cytokines can be activated by ischaemia-reperfusion injury and interaction of AGEs and RAGE [28]. Apoptosis of pericytes has been reported to be associated with inflammation [19]. Additionally, pro-inflammatory cytokines (IL-6 and TNF-α) in the retinal vessels can active pro-apoptosis signalling pathways and promote apoptosis of retinal capillary cells [29]. A study by Kowluru et al. demonstrated the importance of inflammation in retinal pericycle apoptosis by detecting pro-inflammatory cytokines in vitro [30]. Izumi-Nagai et al. and Suzuki et al. reported that AST could inhibit NF-κB activation and downregulate inflammatory cytokines in mice with choroidal neovascularization and uveitis [15, 16]. Park et al. confirmed that AST could reduce inflammatory cytokines and C-reactive protein in plasma, enhancing the cytotoxic activity of natural killer cells and enhancing the immune response in young healthy women [17]. Another study reported that AST reduced NF-κB-mediated inflammation in high-fructose and high-fat diet-fed mice [31]. Previously, anti-inflammatory mechanisms of AST have been reported both in vitro and in vivo. AST reduced the release of inflammatory factors, including IL-6 and TNF-α, as detected using ELISA and Western blot, in a model of hepatic ischaemia reperfusion [32]. Pro-inflammatory cytokines, such as TNF-α, can promote the activation of caspase-3, which can induce apoptosis in retinal endothelial cells [33]. In the present study, it was observed that AST could decrease levels of IL-6, TNF-α, and caspase-3 in the DM+AST group at both protein and mRNA levels, compared to levels in the DM group. This suggests that AST can inhibit development of DR and apoptosis of retinal pericytes by reducing levels of pro-inflammatory cytokines. Present data showed that these indices were upregulated in all diabetic rats, highlighting the relationship between pericycle apoptosis and inflammation.

However, whether AST affects pericycle apoptosis by inhibiting both AGE formation and inflammation or only inhibiting AGE formation remains uncertain. Another limitation of this study was that only a single dose of AST (20 mg/kg/day) was used, based on previous findings. It would be of interest to conduct a dose-response to identify the optimal dose of AST for treatment of STZ-induced diabetic rats. AST could not prevent the destruction of pancreatic β cells induced by STZ in the type 1 diabetes model, as indicated by its inability to prevent hyperglycaemia in the rats. Despite this limitation, AST was still able to afford retinal protection. Protective effects of AST in type 2 diabetes models could be even greater and are of interest. These issues will be addressed in future studies.

Conclusion

The present study demonstrated that administration of AST to STZ-induced diabetic rats partially ameliorated adverse retinal changes that occurred in diabetic rats. These structural changes were likely the result of reduced AGE production, pericyte apoptosis, and inflammation. Although AST could not prevent rises in blood glucose induced by STZ, its ability to protect the eye, at least partially, from the ravages of diabetes suggests that it holds great promise as a therapeutic agent in diabetic patients.

Disclosure of conflict of interest

None.

Abbreviations

DR, diabetic retinopathy; BRB, blood-retina barrier; AGE, advanced glycation end product; AST, astaxanthin; STZ, streptozotocin; IL-6, interleukin-6; TNF-α, tumour necrosis factor-α; NF-κB, nuclear factor-κB; E/P, endothelial cell to pericyte ratio; PBS, phosphate-buffered saline; DAB, 3,3’-diaminobenzidine; SD, standard deviation; RAGE, advanced glycation end-product receptor; IOD, integrated optical density.

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Abstract: Stress, protein aggregation, and loss of functional properties of cells have been shown to contribute to several deleterious pathologies including cancer and neurodegeneration. The incidence of these pathologies has also been shown to increase with age and are often presented as evidence to the cumulative effect of stress and protein aggregation. Prevention or delay of onset of these diseases may prove to be unprecedentedly beneficial. In this study, we explored the anti-stress and differentiation-inducing potential of two marine bioactive carotenoids (astaxanthin and fucoxanthin) using rat glioma cells as a model. We found that the low (nontoxic) doses of both protected cells against UV-induced DNA damage, heavy metal, and heat-induced protein misfolding and aggregation of proteins. Their long-term treatment in glioma cells caused the induction of physiological differentiation into astrocytes. These phenotypes were supported by upregulation of proteins that regulate cell proliferation, DNA damage repair mechanism, and glial differentiation, suggesting their potential for prevention and treatment of stress, protein aggregation, and age-related pathologies.

Keywords: marine carotenoid; ultraviolet radiation; DNA damage; protein misfolding; protein aggregation; glial differentiation; protection

1. Introduction

Stress has been largely defined as a causative factor for a variety of diseases and premature aging as marked by the early occurrence of age-related decline in body functions [1]. On these premises, there is a continuous search and enrollment of natural compounds with anti-stress potential for extending quality of life in normal and disease scenarios. Cell-based functional assays provide a useful model system to identify such physiologically relevant compounds [2]. Oxidative and UV-induced stresses are some of the most common stresses that cause molecular damage both at the DNA and protein level [3], and are often linked to the lethal pathologies including cancer and brain dysfunctions [1,4]. Prevention against stress-induced molecular damage may be useful for disease prevention and/or treatment. Furthermore, assignment of functional properties (such as differentiation to perform specific functions) to cancer cells, that most frequently lose their functional characteristics and de-differentiate
to divide indefinitely, could prove to be a highly useful nontoxic way of treating cancer [5]. In view of this, we chose glioma that possesses the capability to get differentiated into glia/astrocytes. The latter is composed abundantly in intracranial soft tissue, and functions as a major supporting tissue serving anchorage, nourishment, oxygenation, insulation, and excretion to the neurons and various other brain functions. Furthermore, glioma is one of the lethal cancers with a poor prognosis and survival rate [6]. Whereas glial differentiation is well marked by upregulation of GFAP (glial fibrillary acidic protein), PSD95 (postsynaptic density protein 95), MAP2 (microtubule-associated protein 2), and GAP43 (growth-associated protein 43) proteins, glioma often shows either downregulation or lack of these proteins [7–10]. Natural compounds that could induce the upregulation of these proteins and induce glial differentiation have attracted a lot of attention in research laboratories due to their easy availability, relatively lower toxicity index, and economic aspects [11,12].

Some of the relatively unexplored and reasonably newer varieties of natural molecules are those produced in marine organisms. Marine bacteria, fungi, algae, and archaea are healthy and rich sources of certain carotenoids. The latter are either carotenes (pure hydrocarbons) or xanthophylls (oxygenated hydrocarbons) that contribute to distinctive pigmentation of the marine organisms [13]. Astaxanthin and fucoxanthin, as shown in Supplementary Figure S1, are two xanthophylls that are known for their antioxidant and cytoprotective functions [14], and therefore have gained attention in the health, food, and cosmetic industries. Astaxanthin, commonly found in Haematococcus pluvialis and Phaffia rhodozyma, has been shown to possess potent antioxidant and anti-inflammatory activities [14–16]. It showed reactive oxygen species (ROS) and free radical scavenging properties as a quencher of singlet reactive oxygen, nitrogen, as well as single and two electron oxidants. Astaxanthin has also been shown to offer protection against Alzheimer’s disease [17], brain injury [18], cardiac injury [19], contrast-induced nephropathy [20], and muscle atrophy [21]. It is not synthesized by the human body and needs to be obtained exogenously. Since its over-consumption has not been known to cause any toxicity, it has been approved and widely marketed by the United States Food and Drug Administration as a dietary supplement [22,23]. Fucoxanthin, on the other hand, characterized by its distinctively strong orange tint and commonly found in Phaeophyceae and Bacillariophyta, has also been shown to possess similar therapeutic properties [24,25]. However, it was found to be unstable and easily degraded by light, heat, and oxygen [26]. Nevertheless, it has been shown to inhibit proliferation in gastric cancer cells [27], induce autophagy followed by apoptosis in gastric cancer cells at a significantly higher dose [28], and possess potent anticancer effects against glioma cells [29] at a relatively wider range of doses. Fucoxanthin, at higher doses, causes apoptosis and inhibition in cell proliferation by suppression of the PI3K/Akt/mTOR survival pathway and inhibition of their metastatic profiles at relatively lower doses. Similarly, it also protected neurons against ischemic/reperfusion injury [30] and liver cancer cells against tributyltin-induced oxidative injury [31] through the activation of anti-stress heme oxygenase signaling. It has also been shown to protect mice against dextran sulfate sodium-induced colitis [32] and UVB-induced skin erythema [33]. Interestingly, it also extended the overall lifespan in a Drosophila model by triggering distinct genetic modifications [34].

Considering this information, we investigated anti-stress potentials of astaxanthin and fucoxanthin using cell-based biochemical assays. We found that the nontoxic doses of astaxanthin and fucoxanthin protected these cells against UV stress. The treated cells showed astrocytic differentiation characteristics. The reversal of the DNA damage, protein misfolding, and protein aggregation stresses was also observed in cells treated with astaxanthin and fucoxanthin suggesting their potential in the treatment of old-age related pathologies that involve cumulative DNA and protein damage.

2. Results

2.1. Toxicity Profile of Astaxanthin and Fucoxanthin in Rat Glioma

We first examined the toxicity of astaxanthin (Asta) and fucoxanthin (Fuco) on rat glioma cells. As shown in Figure 1A, astaxanthin caused toxicity at doses >200 μM. On the other hand, fucoxanthin
treated cells showed toxicity at doses 8 µM and above. Approximate IC_{50} and IC_{10} and IC_{01} doses, as derived from three independent experiments, are tabulated in Figure 1B. Based on these data, 2.5 µM Asta (A1) and 0.25 µM Fuco (F1) were chosen as the nontoxic doses. Both A1 and F1 treated cells showed no significant difference, with respect to control cells, both on short- and long-term viability, clonogenicity as well as cell morphology as observed by a quantitative cell viability assay [2] and cell proliferation assay, as shown in Figure 1C,D. Therefore, these nontoxic doses were used for further experiments.

![Asterix and Fucoxanthin](image)

**Figure 1.** In vitro toxicity profiling of the marine carotenoids. (A) Dose-dependent cytotoxicity of astaxanthin and fucoxanthin in C6 cells as determined by cell viability assays. (B) IC_{01}, IC_{10}, and IC_{50} doses of astaxanthin and fucoxanthin as determined by dose-dependent cytotoxicity titration curves. (C) Long-term cell proliferation analysis with the safe doses of astaxanthin (A1) and fucoxanthin (F1) showing no effect on proliferation or cell morphology of C6 cells. (D) Quantitative cell viability (QCV) assay, depicting insignificant inhibition of clonogenicity or intracellular stress at the safe dose of both astaxanthin and fucoxanthin. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

### 2.2. Nontoxic Doses of Astaxanthin and Fucoxanthin Protected Cells against DNA Damage Stress

C6 cells were subjected to UV and their IC_{10-30} doses were determined by several independent experiments, as shown in Figure 2A. Next, UV (IC_{10}) treated cells were further treated with Asta or Fuco. As shown in Figure 2B, 5 mJ/cm² of UV radiation caused about 30–50% loss in cell viability over a period of 48 h. Notably, although to a small extent, both Asta and Fuco treatment caused significant recovery with pretreatment, as shown in Figure 2B (left panel), or without pretreatment, as shown in Figure 2B (right panel). UV radiation induces double-strand DNA damage and mutagenesis [35]. A comet assay—a standard method to analyze DNA damage—was performed to check the extent of UV-induced DNA damage and its potential protection by Asta and Fuco. As shown in Figure 2C, 3 mJ/cm² of UV radiation caused considerable (about 18-fold) DNA damage in C6 cells that was significantly limited by both Asta and Fuco supplementation before or after the exposure. In order
to address the mechanism of such protection, we next examined the expression of proteins related to proliferation and DNA damage in control and treated cells. Cells stressed with UV and recovered in control/Asta/Fuco supplemented medium were harvested for immunoblotting and immunostaining for various proteins using specific antibodies. As shown in Figure 3A,B, exposure to 3 mJ/cm² UV radiation caused downregulation of MRN complex, Chk1/2 activation, HP1γ, and mortalin, and upregulation of DNA damage markers 53BP1 and phosphorylated ATR. Cells that were recovered in Asta or Fuco supplemented medium showed significant recovery in MRE11 expression. Furthermore, increase in DNA damage markers (pATR and 53BP1) was abrogated. An immunofluorescence assay confirmed these data and also demonstrated an increase in DNA damage signifying proteins γH2AX, p53, and its downstream PARP1 in cells exposed to UV; the increase was attenuated by Asta or Fuco treatment. Rad50, NBS1, Chk1, Chk2, HP1γ, and mortalin did not show significant changes.

**Figure 2.** Low nontoxic doses of Asta/Fuco protected C6 cells against UV-induced DNA damage. (A) Effect of UV radiation on the viability of C6 cells. (B) UV-responsive cell viability assay showing, small but significant, increase in viability of treated cells; cells pretreated with Asta/Fuco showed stronger effect (left) as compared to the ones treated only after the UV exposure (right). (C) Neutral comet assay showing protection against UV-induced DNA damage in cells treated with Asta/Fuco. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from at least three independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.
Figure 3. Effect of low nontoxic doses of Asta/Fuco on proteins involved in UV-induced DNA damage signaling. Immunoblotting (A) and immunostaining (B) of MRN complex and DNA damage response proteins in control and treated cells. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from at least three independent experiments, and shown as *p < 0.05, **p < 0.01, ***p < 0.001, ns = not significant.

2.3. Nontoxic Doses of Astaxanthin and Fucoxanthin Prevented Protein Aggregation and Protein Misfolding

DNA damage and protein aggregation are the key hallmarks of several diseases including several old age-related brain pathologies. We next examined the effect of Asta and Fuco on protein aggregation using metal-induced protein aggregation as the model [36]. C6 cells were treated with a nontoxic (IC_{10}) dose of sodium (meta)arsenite, as shown in Figure 4A. In order to record the protein aggregation visually, cells were tagged with GFP. As shown in Figure 4B, treated cells showed microscopically appreciable aggregation of GFP. Of note, pretreatment of cells with Asta and Fuco showed clear abolishment of aggregated GFP whereas recovery of cells in the presence of Fuco was equally effective. The aggregates of GFP seen in the cytoplasm of the stressed cells were seen to disappear (deaggregate) when they were treated with Asta or Fuco. Aggregation of the proteins is a common phenomenon found in the pathogenesis of various chronic diseases. We next confirmed such effect of Asta and Fuco using heat-shock-induced protein misfolding of luciferase assays. Cells were transfected with a luciferase-expressing plasmid. Misfolding of luciferase was induced by heat-shock treatment [37]. The effect of Asta and Fuco on protein misfolding was determined quantitatively by luciferase assays. Cells were also immunostained with anti-luciferase antibody to record the expression level and distribution of luciferase. As shown in Figure 4C, heat shock inhibited the expression of luciferase that was significantly reversed with the treatment of Asta and Fuco.
2.4. Nontoxic Doses of Astaxanthin and Fucoxanthin Induced Differentiation in Glioma Cells

Induction of differentiation in brain cancer cells is an important aspect of the therapeutic rationales. We, therefore, investigated the differentiation-inducing potential of Asta and Fuco. As shown in Figure 5A, treatment of C6 cells with Asta and Fuco (the IC₀₁ doses for 30 days) caused the emergence of cells that appeared like differentiated astrocytes (radiating dendrites, soma hypertrophy, process thickening, and axonal reconnections) [38,39]. Of note, cells stressed with either exposure to sodium (meta)arsenite (SMA) or heat shock also showed similar differentiation upon subsequent culture in Asta/Fuco supplemented medium, as shown in Figure 5A,B. There was no significant loss of cell viability observed, as shown in Figure 5B. In order to confirm the astrocytic differentiation, at the molecular level, we examined the expression of protein markers specific to differentiation. As shown in Figure 6A,B, expressions of GFAP, neuropsin, NF200, survivin, vimentin, PSD-95, nestin, MAP2, GAP43, and NCAM proteins were significantly upregulated. RT-PCR analyses revealed that MAP2, GAP43, and nestin were upregulated at the transcriptional level in Asta-treated cells, as shown in Figure 6C. Fuco-treated cells, on the other hand, showed transcriptional upregulation of MAP2 and GAP43; nestin showed a slight decrease. These data strongly suggest that astaxanthin and fucoxanthin have significant glial cell differentiating capacity and may work through common, as well as different, signaling pathways.
Figure 5. Low doses of astaxanthin and fucoxanthin caused glial cell differentiation (A) and its quantification (B) for cell viability and differentiation. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as *** p < 0.001, ns = not significant.
while the acute exposure to oxidative and DNA damaging ultraviolet radiation causes the production of ROS and double-stranded breaks in the DNA. Anomalies in DNA damage repair mechanisms often cause mutations and lead to the development of cancer, early aging, and several degenerative diseases. One of the most commonly explained mechanisms of action of stress to disease transformation is via protein misfolding causing structural changes in the cytoskeleton [37]. It has been firmly established that while the acute exposure to oxidative and DNA damaging ultraviolet radiation causes the production of ROS and double-stranded breaks in the DNA, anomalies in DNA damage repair mechanisms often cause mutations and lead to the development of cancer, early aging, and several degenerative diseases.

Carotenoids from several marine organisms such as microalgae and diatoms are considered and have previously been shown to possess excellent anti-oxidant properties [14,16,24]. Marine carotenoids sources confer with numerous advantages viz., low cost, easy availability, high chemotherapeutic and nutritional index, and low contamination due to their halophilic habitats [40]. Several carotenoids have been isolated and utilized in the pharmaceutical, nutraceutical, and cosmeceutical industries. Understanding their molecular mechanism of action is vital in order to comprehend and rehearse their sustainable and organized use.

Astaxanthin and fucoxanthin are two marine carotene xanthophylls that have been shown to possess powerful anti-oxidant activity owed to their unique structure (epoxide groups, allenic bonds, conjugated double bonds, and conjugated carbonyl groups in the polyene chain) and high potential to quench singlet oxygen and hence act as free radical scavengers [41]. Based on this, they have been predicted to possess preventive and therapeutic potential for oxidative stress-mediated diseases [14,40]. The latter are marked by increased production and accumulation of reactive oxygen species (ROS) that possess the ability to damage cells by oxidizing DNA, proteins, and lipids and worse, mutate them. One of the most commonly explained mechanisms of action of stress to disease transformation is via protein misfolding causing structural changes in the cytoskeleton [37]. It has been firmly established that while the acute exposure to oxidative and DNA damaging ultraviolet radiation causes the production of ROS and double-stranded breaks in the DNA, anomalies in DNA damage repair mechanisms often cause mutations and lead to the development of cancer, early aging, and several degenerative diseases.

**Figure 6.** Expression analysis of key regulators of glial differentiation in control, Asta- and Fuco-treated cells. (A,B) Immunoblotting and immunostaining of cells, showing upregulation differentiation-associated proteins in treated cells. (C) RT-PCR analysis, showing that MAP2 and GAP43 proteins are transcriptionally upregulated in treated cells; nestin showed upregulation in Fuco-treated cells only. Statistical significance was calculated by an unpaired *t*-test of GraphPad® software (2018) using mean, SD, and N from at least three independent experiments, and shown as * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001.

### 3. Discussion

Carotenoids from several marine organisms such as microalgae and diatoms are considered and have previously been shown to possess excellent anti-oxidant properties [14,16,24]. Marine carotenoids sources confer with numerous advantages viz., low cost, easy availability, high chemotherapeutic and nutritional index, and low contamination due to their halophilic habitats [40]. Several carotenoids have been isolated and utilized in the pharmaceutical, nutraceutical, and cosmeceutical industries. Understanding their molecular mechanism of action is vital in order to comprehend and rehearse their sustainable and organized use.

Astaxanthin and fucoxanthin are two marine carotene xanthophylls that have been shown to possess powerful anti-oxidant activity owed to their unique structure (epoxide groups, allenic bonds, conjugated double bonds, and conjugated carbonyl groups in the polyene chain) and high potential to quench singlet oxygen and hence act as free radical scavengers [41]. Based on this, they have been predicted to possess preventive and therapeutic potential for oxidative stress-mediated diseases [14,40]. The latter are marked by increased production and accumulation of reactive oxygen species (ROS) that possess the ability to damage cells by oxidizing DNA, proteins, and lipids and worse, mutate them. One of the most commonly explained mechanisms of action of stress to disease transformation is via protein misfolding causing structural changes in the cytoskeleton [37]. It has been firmly established that while the acute exposure to oxidative and DNA damaging ultraviolet radiation causes the production of ROS and double-stranded breaks in the DNA, anomalies in DNA damage repair mechanisms often cause mutations and lead to the development of cancer, early aging, and several degenerative diseases.
Chronic exposure to these stresses leads to continual protein misfolding and aggregation [42,43]. These aggregates contribute to age-related degenerative pathologies to a large extent [1,44–46]. Most recently, cancer has been defined as one of the old-age related pathologies, partly due to extended human life-span and increased level of chemical and environmental stresses [47]. Cancer diagnostics and therapeutics have made remarkable progress in the last two to three decades [48]. A large number of anticancer drugs have traveled from the laboratory to clinics. However, most of the drugs are synthetic and are associated with severe adverse effects and drug resistance. To overcome this problem, the discovery and development of several alternate means of anti-stress therapies are on the rise. One of the recently introduced targeted therapies applicable to brain-related cancers is the induction of cancer cell differentiation [5]. Owing to the high prevalence of brain cancers, the high rate of carcinogenic de-differentiation in brain tissues, and their failure to regenerate after chemotherapy-mediated cytotoxicity, cancer cell differentiation may be considered one of the most rewarding antitumor remedies.

Comparable to the conventional remedies, astaxanthin and fucoxanthin showed both anti-stress and differentiation-inducing potential in rat glioma cells. Of note, stress (radiation, temperature, and heavy metal poisoning)-induced molecular changes were reversed in some, if not all, when cells were treated with astaxanthin and fucoxanthin. Ultraviolet radiation has been shown to cause indirect damage to the DNA via absorption of photons by the non-DNA chromophores and a resultant ROS formation that oxidize DNA bases causing mutations [49], triggering the DNA damage response signaling pathway orchestrated by the key regulator ATR kinase recruiting proteins such as the MRN complex, 53BP1, and BRCA, and functional repression of proliferation via the expression of HP1γ [50–52]. We found that the culture of UV-stressed C6 cells in astaxanthin- or fucoxanthin-supplemented medium led to the suppression of the DNA damage markers pATR and 53BP1, and partial reactivation of the MRN complex, as shown in Figure 3. The mechanism of action of protection against UV radiation by astaxanthin and fucoxanthin in glioma cells could be attributed to their ability to reactivate the MRN complex and its downstream factor 53BP1, leading to the reciprocal phosphorylation of ATR, thereby facilitating the process of DNA damage repair inhibition, as shown in Figure 2B,C. On the other hand, HP1γ and mortalin (chromatin modulating and stress chaperone proteins, respectively) showed a decrease in UV-stressed control as well as treated cells suggesting that astaxanthin and fucoxanthin may work predominantly through protecting the cells against DNA damage [53]. The latter has been implicated in chronic protein aggregating degenerative diseases [54–56]. Heavy metals have been known to interfere with protein homeostasis and stabilization, via formation of toxic P-bodies (or stress granules) that aggregate and co-sediment with several heat-shock chaperones leading to the protein misfolding [36,57]. Proteinopathies resulting from long-term protein misfolding and/or failure of the cells to eliminate misfolded proteins have been known to be one of the major causes of old-age related pathologies [58]. A number of neurodegenerative diseases are marked by the hallmark events of protein misfolding, and their aggregation and accumulation that further cause a loss of synaptic connections and cellular dysfunction in brain tissue. Both astaxanthin and fucoxanthin caused deaggregation of the sodium (meta)arsenite-induced aggregated GFP protein, as shown in Figure 4A,B, and inhibited heat-shock-induced folding of luciferase protein, as shown in Figure 4C. Our results suggest the plausible ability of astaxanthin and fucoxanthin to protect the cells against heavy metal or heat-induced stress including protein aggregation and molecular damage, thereby useful against protein aggregation/misfolding mediated pathologies.

Astaxanthin and fucoxanthin both strongly caused differentiation in C6 cells into the respective functional phenotypes (as supported by molecular changes), signifying that they could serve as important and attractive differentiation-based therapeutic agents. Cells treated with astaxanthin and fucoxanthin showed characteristics of astrocytes including radiating dendrites, flattened morphology, soma hypertrophy, and cell-to-cell connections with synaptic junctions [38,39]. Molecular analysis indeed revealed upregulation of proteins involved in functional neurogenesis. The differentiated cells demonstrated a distinct upregulation pattern in the expression of MAP2 (morphogenesis regulator...
and organelle trafficker) and PSD-95 (post-synaptic targeting scaffold) [59], GAP43 (key protein in growth cone formation, neurite outgrowth, and development of functional cerebral cortex) [60], GFAP and vimentin (shape and motility modulators) and nestin (neural cell division and migration regulator) [61], neurexin (functional photoreceptor) [62], NCAM (responsible for neuronal attachment, extension, and cell-to-cell interaction) [63] and survivin (cell survival and proliferation enhancer) [62] proteins. These signature proteins may also endorse the reconstruction of glioma cells into mature neuronal and Schwann cell phenotypes, as shown in Supplementary Figure S2. These data suggest that the marine carotene xanthophylls, astaxanthin and fucoxanthin, possess potent anti-stress, anti-protein aggregation, and differentiation-inducing activities that warrant further mechanistic investigations for their recruitment in stress/disease prevention and therapeutics.

4. Materials and Methods

4.1. Cell Culture and Reagents

Rat glioma cells (C6) (obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen)-supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator (37 °C and 5% CO₂). The marine carotenoids, astaxanthin (Supreme Health New Zealand Limited, Auckland, New Zealand; Batch # SB10012102013B; mol. weight 596.84 containing 5% free and 95% di/mono ester forms) and fucoxanthin (Wako, 063-06691; CAS number 3351-86-8; mol. weight 658.91, 95% purity by weight) were dissolved in DMSO to make 100 mM and 5 mM stocks, respectively, and stored at −20 °C. Sodium (meta)arsenite was dissolved in ultrapure water to make a 100 mM stock and stored at 4 °C. A UV chamber (FS-800, FUNA®-UV-linker) was used to induce UV-radiation stress. Antibodies against luciferase (Abcam, Cambridge, UK, ab164664), GFAP (Sigma-Aldrich, St. Louis, MO, USA, G9269), NF200 (Sigma-Aldrich, N4142), PSD95 (Santa Cruz, Dallas, TX, USA, sc-32290), MAP2 (Sigma-Aldrich, M3696), nestin (Santa Cruz, sc-23927), neurexin (Santa Cruz, sc-134600), GAP43 (Santa Cruz, sc-33705), NCAM (Santa Cruz, sc-10735), survivin (Santa Cruz, sc-10811), vimentin (Santa Cruz, sc-6260), γH2AX (Cell Signaling, Danvers, MA, USA, 9718S), HP1γ (Merck Millipore, Burlington, MA, USA, 05-690), pChk1 (Cell Signaling, 2344S), pChk2 (Cell Signaling, 2197P), mortalin [64], p53 (Santa Cruz, SC-126), MRE11 (Novus Biologicals, Centennial, CO, USA, NB100-142), Rad50 (Cell Signaling, 3427S), NBS1 (Abcam, ab32074), pATR (Santa Cruz, sc-109912), PARP1 (Santa Cruz, sc-7150), 53BP1 (Abcam, ab21083), BRCA (Cell Signaling, 9010S), and β-actin (Abcam, ab49900) proteins were used for immunoblotting and immunostaining. Primers for MAP2 (F-CTGCACTTTTATTTTACCACTTCCT/R-CGCTTATGCCCATTCTTC), nestin (F-GAACATCGATGCTCAGATCC/R-GCAGCACACCAGATAAGCT), GAP43 (F-ATGCTGTGCTGTATGAGAAGAACC/R-GCAGCACACCAGATAAGCT), and GAPDH (F-TGGAAATCCCATCACCATCT/R-TTCACACCCATGACGAACAT) were used for mRNA estimation in reverse transcriptase polymerase chain reaction.

4.2. Dose Titration

Cytotoxicity of the astaxanthin, fucoxanthin, sodium (meta)arsenite, and UV radiation in C6 cells were determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Two thousand cells per well were plated in a 96-well plate, allowed to settle overnight, and treated with varying doses of the compounds/stressors. The control (DMSO) or treated cells were incubated for 48 h followed by incubation of 10 μL of phosphate buffered saline (PBS) containing 5 mg/mL MTT (M6494, Life Technologies, Carlsbad, CA, USA), and further incubated for 4 h. Culture medium containing MTT was aspirated and replaced with DMSO. The plates were placed on a shaker for 5 min followed by measurement of optical density at 570 nm using Tecan infinite M200® Pro microplate reader (Tecan Group Ltd., Mannedorf, Switzerland). Cell viability was calculated as a percentage against the control to identify their IC₀₁ value using Microsoft™ Office© 2016. Statistical significance was calculated by
an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

4.3. QCV Assay

Five hundred cells per well were plated in a 6-well plate and allowed to settle overnight, followed by treatment with varying doses of the marine carotenoids. The control or drug-treated cells were incubated at 37 °C and 5% CO₂. The drug-supplemented medium was replaced every alternate day. After 8 days, cells were fixed, stained, and de-stained into the solution, which was quantified by the help of a spectrophotometer as described previously [2]. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

4.4. Cell Proliferation

In order to estimate the proliferative rate of cells, five thousand cells were plated in 6-well plates and allowed to settle overnight, followed by culture either in control or drug-supplemented medium. Cells were fixed in each variant of treatment and counted every day for 8 days. A proliferation histogram was plotted considering control on day 1 as 100 percent using Microsoft™ Office 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as *** p < 0.001, ns = not significant.

4.5. Cell Differentiation and Crystal Violet Staining

Two thousand cells per well were plated in a 6-well plate, allowed to settle overnight, and treated with varying doses of the compounds/stressors. The drug-supplemented medium was replaced every alternate day for 28 days. Cells were then visualized under a phase contrast microscope and recorded at X400 magnification. Differentiated cells were counted manually as random 100 selections by ImageJ software 1.52a (NIH), and tabulated and averaged using Microsoft™ Office 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as *** p < 0.001, ns = not significant.

4.6. Stress Protection

Two thousand cells per well were plated in two 96-well plates and allowed to settle overnight. Cells in the first plate were pretreated with varying doses of the compounds/stressors. The control (DMSO) or treated cells were incubated for 24 h, following which the cells in both the plates were stressed with UV stress (a well-established model of DNA and oxidative damage) at the rate of 5 mJ/cm² and recovered in either control or drug-supplemented medium for 48 h (plate 1 = pretreatment model, plate 2 = recovery model), followed by addition of 10 μL of phosphate buffered saline (PBS) containing 5 mg/mL MTT (M6494, Life Technologies), and further incubated for 4 h. Culture medium containing MTT was aspirated and replaced with DMSO. The plates were placed on a shaker for 5 min followed by measurement of optical density at 570 nm using Tecan infinite M200® Pro microplate reader (Tecan Group Ltd., Mannedorf, Switzerland). Cell viability was calculated as a percentage against the control using Microsoft™ Office 2016. The experiment was performed three times, and the histograms bearing cumulated data were plotted. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from four independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

4.7. Comet Assay

One hundred thousand cells per well were plated in a 6-well plate, allowed to settle overnight, and pretreated with varying doses of the compounds. After 24 h of pretreatment, the cells were stressed with UV radiation at the rate of 3 mJ/cm², with 50 mJ/cm² as the positive control, and recovered in
the drug-supplemented medium. The protective effect of the marine carotenoids against UV-mediated DNA damage was evaluated using a single cell gel neutral comet assay (Trevigen’s Comet Assay®) electrophoresis system following the manufacturer’s protocol. Comet tail length was calculated by ImageJ software (NIH) and tabulated as a percentage against the control using Microsoft™ Office© 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as *** p < 0.001.

4.8. Generation of Stable GFP-Expressing Cells

Five thousand cells per well were plated in a 12-well plate and allowed to settle overnight. Cells were transfected with 100 ng of plasmid-expressing GFP from a constitutive (β-actin) promoter using Lipofectamine™ 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA, 11668027) in Opti-MEM™ reduced serum medium (Gibco™, 10149832). Forty-eight hours later, the transfection efficiency was determined by direct observation under the microscope. The cells with more than 50% transfection were processed through selection with Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 0.5 mg/mL hygromycin B (Clontech, Mountain View, CA, USA, 8057-1) for 96 h. Colonies of GFP-expressing C6 cells were then identified and marked under the microscope, transferred onto a fresh culture dish manually, and allowed to grow under favorable conditions to form a stable GFP-expressing C6 cell line.

4.9. Protein Aggregation and Deaggregation

Fifty thousand GFP-expressing C6 cells per well were plated in a 6-well plate, allowed to settle overnight, and pretreated with varying doses of the compounds. After 24 h, the cells were stressed with sodium (meta)arsenite 20 μM for another 24 h, following which they were washed thoroughly with PBS thrice and recovered in the drug-supplemented medium for 48 h. Cells were then visualized under a fluorescent microscope and recorded at 400× magnification. Aggregates were quantified using ImageJ software (NIH) and plotted as a percentage using Microsoft™ Office © 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) in stressed-treated samples using mean, SD, and N from four independent experiments against the positive control, and shown as ** p < 0.01, *** p < 0.001.

4.10. Heat-Shock Aggregation of Luciferase

One hundred thousand cells per well were plated in a 6-well plate in two identical sets and allowed to settle overnight. The cells were transfected with pGL4-p53-3’ UTR expressing luciferase from a constitutive promoter as described earlier. After 48 h, cells were heat shocked at 42 °C and 5% CO₂ for 2 h, followed by recovery at 37 °C either in the control or drug-supplemented medium for the next 48 h. The first set of cells were taken for immunocytochemistry for luciferase protein as described later in Section 4.12, and the second was lysed using passive lysis buffer for luciferase expression estimation using the luciferase assay system (Promega, Madison, WI, USA, E1501) following the manufacturer’s protocol. Protein expression was quantified by ImageJ software (NIH) and tabulated with reporter luciferase luminescence values as a percentage against the control using Microsoft™ Office© 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as *** p < 0.001.

4.11. Immunoblotting

Two hundred thousand cells per well were plated in a 6-well plate and allowed to settle overnight, followed by the treatment with varying doses of the compounds/stressors. Control and treated cells were harvested and washed with PBS (X2), followed by lysis in RIPA buffer (89900, Thermo Fisher Scientific) containing complete protease inhibitor cocktail (4693159001, Roche Applied Science, Penzberg, Bavaria, Germany) on ice for 45 min. Lysates were separated on an SDS-polyacrylamide gel using Mini-Protean® Tetra cell equipment (Bio-Rad, Hercules, CA, USA), and subjected to
western blotting using protein-specific antibodies as indicated and horseradish peroxidase-conjugated secondary HRP antibody (31430 or 31460, Thermo Fisher Scientific). Blots were developed using chemiluminescence solution (GE Healthcare, Buckinghamshire, UK) and visualized using a Lumino Image Analyzer (LAS 3000-mini; Fuji Film, Tokyo, Japan). Band intensity was quantified using ImageJ software (NIH) and plotted as a percentage using Microsoft™ Office® 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from four independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

4.12. Immunostaining

Twenty-five thousand cells per well were plated on glass coverslips placed in 12-well cell culture plates. After 48 h of treatment with drugs/stressors, control or treated cells were fixed in methanol:acetone (1:1). Cells were permeabilized with Tween-20 in phosphate buffered saline (PBST), washed with phosphate buffered saline (PBS), and blocked with 2% bovine serum albumin protein dissolved in PBST. Fixed cells were incubated with primary antibodies (as indicated) overnight, washed with PBST-PBS-PBS (5 min each), incubated with either Alexa-Fluor 488 goat anti-mouse IgG (Life Technologies, A11029) or Alexa-Fluor 594 goat anti-rabbit IgG (Life Technologies, A11037), depending upon the source of the primary antibodies, for 2 h, washed with PBS-PBST-PBS (5 min each), incubated with Hoechst 33342 stain (Invitrogen Molecular Probes, Carlsbad, CA, USA, H3570) for 10 min, washed with PBST-PBS-ultrapure water (5 min each), and mounted on glass slides. The cells were then visualized for immunofluorescence under a microscope at 400× magnification. Protein expression was quantified using ImageJ software (NIH) and plotted as a percentage using Microsoft™ Office® 2016. Statistical significance was calculated by an unpaired t test of GraphPad® software (2018) using mean, SD, and N from three independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

4.13. Reverse Transcriptase Polymerase Chain Reaction

Two thousand cells per well were plated in a 6-well plate, allowed to settle overnight, and treated with varying doses of the compounds/stressors. The drug-supplemented medium was replaced every alternate day for 25 to 35 days until the appearance of differentiated morphology in the cells (described in earlier sections). Cells were harvested from the Petri dishes and lysed with Trizol (Ambion®, Foster City, CA, USA, 15596018) for 5 min at room temperature, segregated in chloroform (Wako, Tokyo, Japan, 038-02606) for 5 min at room temperature, centrifuged at 12,000 rpm for 15 min and supernatant separated, washed in isopropanol (Wako, 166-04836) for 10 min at room temperature, centrifuged at 12,000 rpm for 15 min and pellet washed in 70% ice-cold ethanol and centrifuged at 8000 rpm for 5 min twice, followed by air-drying and resuspension in nuclease-free water to extract pure RNA. The concentration and quality of RNA were evaluated through a spectrophotometer (ND-1000, Nanodrops®, Wilmington, NC, USA). cDNA was prepared using a reverse transcription kit (Qiagen, Hilden, Germany, 205313) following the manufacturer’s instructions. The master mix for amplification was prepared by mixing 1 µL cDNA with 0.1 µL Ex Taq (Takara, Kusatsu, Shiga, Japan, RR001), 2 µL 10× TAQ buffer, 2 µL dNTP, 1 µL each of forward and reverse primers (indicated earlier) in 12.9 µL nuclease free water and amplified using ‘denaturation—95 °C, 10 min → amplification—95 °C, 45 s–60 °C, 1 min−72 °C, 45 s (35–48 cycles) → annealing—72 °C, 10 min → 4 °C’ protocol. The amplified products were separated on a 1% agarose gel containing 0.0625 µg/mL ethidium bromide (Invitrogen®, 15585-011), and acquired using a Lumino Image Analyzer (LAS3000-mini; Fuji Film, Tokyo, Japan) equipped with a CCD (Charge-coupled device) camera. Band intensity was quantified using ImageJ software (NIH) and plotted as a percentage using Microsoft™ Office® 2016. Statistical significance was calculated by an unpaired t-test of GraphPad® software (2018) using mean, SD, and N from four independent experiments, and shown as * p < 0.05, *** p < 0.001.
4.14. Statistical Analysis

All the quantifications were performed using ImageJ software (NIH), calculations were done using Microsoft™ Office © 2016, and plotted as percentages. Statistical significance was calculated by an unpaired t-test of GraphPad ® software (2018) using mean, SD, and N from three independent experiments, and shown as * p < 0.05, ** p < 0.01, *** p < 0.001, ns = not significant.

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/17/3/189/s1, Figure S1. 2-D and 3-D chemical structures of the two selected marine carotenoids, Astaxanthin and Fucoxanthin; Figure S2. Schematic presentation of the effect of Astaxanthin and Fucoxanthin on C6 cell differentiation determining proteins.

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Review

Astaxanthin as a Peroxisome Proliferator-Activated Receptor (PPAR) Modulator: Its Therapeutic Implications

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Abstract: Peroxisome proliferator-activated receptors (PPARs) are part of the nuclear hormone receptors superfamily that plays a pivotal role in functions such as glucose and lipid homeostasis. Astaxanthin (ASX) is a lipid-soluble xanthophyll carotenoid synthesized by many microorganisms and various types of marine life that is known to possess antioxidant, anti-inflammatory, antidiabetic, anti-atherosclerotic, and anticancer activities. As such, it is a promising nutraceutical resource. ASX-mediated modulation of PPARs and its therapeutic implications in various pathophysiological conditions are described in this review. ASX primarily enhances the action of PPARα and suppresses that of PPARβ/δ and PPARγ, but it has also been confirmed that ASX displays the opposite effects on PPARs, depending on the cell context. Anti-inflammatory effects of ASX are mediated by PPARα activation, which induces the expression of pro-inflammatory cytokines in macrophages and gastric epithelial cells. The PPARα-agonistic effect of ASX treatment results in the inhibition of cellular growth and apoptosis in tumor cells. Simultaneous and differential regulation of PPARα and PPARγ activity by ASX has demonstrated a hepatoprotective effect, maintaining hepatic lipid homeostasis and preventing related hepatic problems. Considering additional therapeutic benefits of ASX such as anti-gastric, cardioprotective, immuno-modulatory, neuroprotective, retinoprotective, and osteogenic effects, more studies on the association between ASX-mediated PPAR regulation and its therapeutic outcomes in various pathophysiological conditions are needed to further elucidate the role of ASX as a novel nutraceutical PPAR modulator.

Keywords: astaxanthin; peroxisome proliferator-activated receptors (PPARs); anti-inflammation; anticancer; lipid and glucose metabolism; PPAR modulator

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptors superfamily and are ligand-activated transcription factors [1]. They play an important role in the expression of many genes regulating cellular differentiation, the metabolism of glucose and lipids, and carcinogenesis [2–4]. Since the first PPAR was discovered in 1990 [5], three isoforms (PPARα, β/δ, and γ) have been identified in mammalian species [6]. PPARα is mainly expressed in the liver, kidney, heart, and skeletal muscle and is mainly involved in lipid metabolism and insulin sensitivity [7,8]. PPARβ/δ is ubiquitous throughout the human body and is responsible for epithelial cell growth, fatty acid oxidation, and wound healing, [9,10]. PPARγ is the most studied subtype and is found primarily in adipose tissues. In addition to its major role in glucose and lipid homeostasis, PPARγ is also associated with inflammatory responses, cardiovascular diseases, neurogenerative diseases, ocular diseases, and cancer [11]. Since these various physiological functions of PPARs can serve as therapeutic targets for the treatment of chronic diseases, researchers have studied synthetic and
naturally occurring substances, as well as marine organisms, to identify specific ligands that modulate PPAR activities [12–21].

Astaxanthin (ASX; 3,3′-dihydroxy-β,β-carotene-4,4′-dione) (Figure 1) is a lipid-soluble, red-orange-colored xanthophyll carotenoid synthesized by many microorganisms and various types of marine life [22]. ASX was first identified from Astacus gammarus (European lobster) in 1938 [23] and was initially approved as a food dye for salmon, trout, and shrimp feed in aquaculture industries [24]. Later in 1999, the United States Food and Drug Administration (USFDA) allowed the use of ASX as a dietary supplement [25]. A green microalga, Haematococcus pluvialis, is known to be a major producer of ASX. It accumulates up to 5% dry weight of ASX during encystment, which includes four progressive cellular morphological phases (Figure 2) [26]. Because synthetic ASX from petrochemicals exhibited inferior effects on human health when compared with natural algal-based ASX [27], many studies have been conducted on maximizing ASX production from H. pluvialis [28–32].

![Chemical structure of astaxanthin (ASX).](image1.png)

**Figure 1.** Chemical structure of astaxanthin (ASX).

![Microscopic appearance in Haematococcus pluvialis encystment.](image2.png)

**Figure 2.** Microscopic appearance in *Haematococcus pluvialis* encystment. (A) green vegetative motile cell; (B) green vegetative palmella cell; (C) palmella cell accumulating ASX; (D) ASX fully-accumulated red aplanospore cell (from Shah et al. [26] distributed under the terms of the Creative Commons Attribution 4.0 International License (CC BY 4.0)).

Numerous studies have demonstrated various biological properties and mechanisms of action of ASX. It displays antioxidant, anti-inflammatory, antidiabetic, anti-atherosclerotic, and anticancer activities and is a promising nutraceutical resource [33–38]. In this article, ASX-mediated PPAR modulation and its therapeutic implications are extensively reviewed and discussed.

2. **Effects of ASX on PPAR Isoforms**

2.1. **PPARα**

Most studies indicate that ASX acts as an agonist to PPARα [39–41]. Jia et al. [39] demonstrated that ASX significantly increased PPARα transactivation efficacy in PPARα-transfected Chinese hamster ovary (CHO-K1) cells; this effect was concentration-dependent. They also showed that ASX exhibits direct binding to human PPARα ligand binding domain with a KD value (concentration at which a compound dissociates from the immobilized protein after the association phase) of 197 μM. The proportional sigmoidal increase in the time-resolved fluorescence resonance energy transfer (TR-FRET) ratio in agonist assay mode (the half-maximal effective concentration (EC50) = 3.9 μM) suggested that ASX plays a role as a ligand to activate PPARα. Furthermore, ASX significantly induced PPARα transcription and affected the expression of related target genes in HepG2 human hepatocellular carcinoma cells. In two other in vivo studies that used animals with a high-fat diet, administration of ASX alone [40] or in a combined treatment with flaxseed oil [41] showed a significant and
dose-dependent increase in hepatic expression of the PPARβ/δ gene and protein. Meanwhile, a recent study [42] reported that ASX is predicted to suppress the expression of PPARs and its target molecules in the livers of mice with diet-induced nonalcoholic steatohepatitis (NASH).

2.2. PPARβ/δ

Because early studies have not identified any significant relationship between ASX and PPARβ/δ expression and/or activity [39,43], follow-up studies have not been conducted consistently. The relevant effect of ASX on PPARβ/δ was first confirmed by Kobori et al. [42] when they discovered that ASX significantly decreased mRNA expression of PPARβ/δ and related target genes in NASH mice. Another study by Rundblad et al. [44] also demonstrated that the intake of high-oleic sunflower oil (HOSO) with added ASX downregulates mRNA expression of genes associated with glucose and lipid metabolism, including PPARβ/δ, in the peripheral blood mononuclear cells (PBMCs) of healthy volunteers. Similar observations were observed in subjects receiving krill oil containing the same amount of ASX.

2.3. PPARγ

The physiological role of ASX in PPARγ expression and activity is quite complex. The association between ASX and PPARγ was first described in 2005 [45] when a specific PPARγ antagonist, GW9662, strongly inhibited ASX-induced expression of connexin 43, a protein related to early processes in carcinogenesis [46]. Similar observations were reported in ASX-treated K562 leukemia cells [47] and AGS cells (human gastric epithelial adenocarcinoma cell line) [48]. ASX significantly and dose-dependently induced cellular apoptosis and PPARγ protein expression in K562 cells, and these effects were attenuated by GW9662 [47]. Most recently, ASX was found to increase the expression and DNA-binding activity of PPARγ in Helicobacter pylori-infected AGS cells in a dose-dependent manner. In those same cells, ASX also improved catalase activity, inhibited intracellular and mitochondrial reactive oxygen species (ROS) levels, and diminished the gene expression of inflammatory cytokines that are suppressed by GW9662 co-treatment [48]. Another study by Kim et al. [49] also showed a significant increase in PPARγ mRNA levels along with other osteogenesis- and adipogenesis-related genes in ASX-treated neural stem cells.

In contrast, other studies have suggested that ASX may be a PPARγ antagonist. Jia et al. [39] reported that ASX dose-dependently inhibited PPARγ transactivation with a more than 16-fold higher K_D value (11.9 μM) compared with the value observed in PPARα. In the TR-FRET coactivator antagonist assay mode, the half-maximal inhibitory concentration (IC50) value for PPARγ was 607.8 μM. In addition, the expression of PPARγ and related genes was also significantly decreased by ASX treatment. Her et al. [50] used the ubiquitous transcription factor Yin Yang 1 (YY1)-transgenic zebrafish lines (GY), which are characterized by their induced expression of lipogenic genes, including PPARγ, associated with CCAAT-enhancer-binding protein (C/EBP) homologous protein 10 (CHOP-10) downregulation. The authors observed a marked decrease in PPARγ expression with preserved CHOP-10 levels in high-level GY-expressing (GY-H) larvae, which is characteristic of normal zebrafish lines. In an in vivo approach, high-dose ASX (30 μM) suppressed PPARγ expression and consequently influenced the mRNA levels of several genes involved in hepatic lipid metabolism in mice fed a high-fat diet [40].

Considering these conflicting outcomes, Inoue et al. [43] advanced a new theory that ASX acts as a selective PPARγ modulator (SPPARM) depending on the cell context. Among the various xanthophyll carotenoids, ASX only showed dose-proportional binding to PPARγ, with less binding affinity and lower maximum activation than the PPARγ full agonist rosiglitazone. Luciferase reporter gene assays using human embryonic kidney 293 (HEK293) cells as well as an evaluation of adipogenesis and target gene expression in 3T3-L1 adipocytes demonstrated that ASX has an antagonistic effect on PPARγ. Meanwhile, ASX treatment increased mRNA and/or protein expression of liver X receptor (LXR) and CD36 in a dose-dependent manner. Subsequently, ASX also increased the induction of ATP-binding cassette transporter ABCA1 and ABCG1 in thioglycollate-elicited peritoneal macrophages, which is
attributable to the action of PPARγ agonists. The authors suggested possible benefits of ASX for the management of various chronic diseases, through the adaptive PPARγ-modulating effect.

3. PPAR-Related Therapeutic Implications of ASX

The biological and pathophysiological activities of ASX due to its regulation of PPARs are summarized in Table 1.

3.1. Anti-Inflammatory Effects

The anti-inflammatory properties of ASX have been described by many previous studies [33,37], and various molecular mechanisms of action have been suggested. These include a blockade of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway [51–56], inhibition of c-Jun N-terminal kinase (JNK) in the mitogen-activated protein kinase (MAPK) signaling pathway [54,56], prevention of ROS accumulation by nuclear factor E2-related factor 2 (Nrf2) [55], positive modulation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) protein expression [51], suppression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [57], and induction of heme oxygenase-1 (HO-1) [58].

Although more investigations are needed to clarify the association between PPARs and inflammation, it has been reported that PPARγ ligands can regulate inflammatory responses by the transrepression of several signaling pathways (NF-κB, activating protein 1 (AP-1), and signal transducer and activator of transcription (STAT)-1) [59]. In agreement with this, ASX showed the induction of LXR and CD36 mRNA expression via PPARγ activation in macrophages [43]. In addition to their involvement in cholesterol and lipid metabolism, LXRs also suppress the expression of pro-inflammatory genes such as tumor necrosis factor α (TNF-α), COX-2, iNOS, and matrix metalloprotease 9 (MMP9) [60]. CD36, a class B transmembrane scavenger receptor, is expressed by multiple cell types including macrophages and plays an important role in the pro-inflammatory and oxidative pathways [61,62]. ASX also exhibited protective effects against *H. pylori*-induced gastric inflammation [48]. *H. pylori* induces the release and activation of inflammatory cytokines, such as interleukin (IL)-8, via NF-κB activation in gastric mucosa [63–65]. Subsequently, IL-8 stimulates the assembly of neutrophils and ROS generation in the infected lesion [66]. PPARγ activation by ASX treatment improved the activity of antioxidant enzyme catalase (a downstream target gene for PPARγ), restored ROS overproduction, and inhibited IL-8 expression in *H. pylori*-infected gastric epithelial cells.

Meanwhile, ASX-mediated reductions in plasma and hepatic TNF-α and IL-6 expression were reported in an in vivo study [40], which suggests the possible involvement of PPARα activation, but not of PPARγ activation.
Table 1. Summary of PPAR-related biological and pathophysiological activities of ASX.

| Biological Activity            | Effect on PPARs | Study Model                              | Study Result(s)                                                                 | References |
|--------------------------------|-----------------|------------------------------------------|--------------------------------------------------------------------------------|------------|
| **Anti-inflammatory**          | PPARγ†          | Thioglycollate-elicited peritoneal macrophages from C57BL/6J mice | Induced mRNA expressions of LXR and CD36                                        | [43]       |
|                                | PPARγ†          | H. pylori-infected AGS human gastric epithelial cells | Inhibition of H. pylori-induced increase in intracellular and mitochondrial ROS levels and IL-8 gene expression | [48]       |
|                                | PPARα†          | High-fat diet-fed C57BL/6J male mice       | Reduced mRNA expression and plasma and liver levels of TNF-α and IL-6             | [40]       |
| **Anticancer**                 | PPARγ†          | C3H/10T1/2 mouse embryonic fibroblast cells | Induction of connexin 43 expression                                              | [45]       |
|                                | PPARγ†          | K562 leukemia cells                       | Cellular growth inhibition, cell cycle arrest and induction of apoptosis          | [47]       |
|                                | PPARα†, PPARγ↓  | Lipid-loaded HepG2 human hepatocarcinoma cells | Reduced cellular cholesterol and triglyceride contents; changes in target gene expressions for PPARα and PPARγ involved in lipid and glucose metabolism pathways | [39]       |
|                                | PPARα†, PPARγ↓  | High-fat diet-fed C57BL/6J male mice       | Altered expressions in several PPARα and PPARγ target genes; reduced hepatic steatosis | [40]       |
|                                | PPARα†          | High-fat diet-fed Sprague-Dawley rats      | Increased mRNA expressions in CPT1 and ACOX; decreased mRNA expressions in SREBP1, HMGCR, FAS, and ACC; reduced hepatic steatosis and hepatic triglyceride and total cholesterol levels | [41]       |
| **Lipid and glucose homeostasis** | PPARγ†          | YY1-transgenic zebrafish                 | Yellow and greasy appearance and marked lipid accumulation in the hepatocytes; increased mRNA expression of genes responsible for the fatty acid synthesis, transport and binding, lipid storage, and hepatic lipogenesis; upregulation of C/EBPα and PPARγ target genes; gross liver hypoplasia and related lipotoxicity | [50]       |
|                                | PPARα↓, PPARβ/δ↓| C57BL/6J mice with high-cholesterol, high-cholate, and high-fat diet-induced NASH | Changes in PPAR target genes (inhibition of PNPLA2; activation of PML)            | [42]       |
|                                | PPARγ↓          | 3T3-L1 adipocytes                        | Inhibition of rosiglitazone-induced lipid accumulation; reduced aP2, FABP, and LPL mRNA levels | [43]       |
|                                | PPARγ↑          | Mouse neural stem cells                  | Increased lipid accumulation; overexpression of adipogenic genes                 | [49]       |
|                                | PPARβ/δ↓        | PBMCs from healthy volunteers            | Downregulation of genes involved in lipid and glucose metabolism (including PPARβ/δ) | [44]       |

ACC, acetyl-CoA carboxylase; ACOX, acyl-CoA oxidase; aP2, adipocyte protein 2; ASX, astaxanthin; CPT1, carnitine palmitoyltransferase 1; FABP, fatty acid binding protein; FAS, fatty acid synthase; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IL, interleukin; LPL, lipoprotein lipase; LXR, liver X receptor; NASH, nonalcoholic steatohepatitis; PBMC, peripheral blood mononuclear cell; PML, promyelocytic leukemia protein; PNPLA2, patatin-like phospholipase domain containing 2; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxygen species; SREBP1, sterol regulatory element binding protein 1; TNF-α, tumor necrosis factor α; YY1, Yin Yang 1 transcription factor.
3.2. Anticancer Effects

ASX has demonstrated anticancer activity through multiple mechanisms including cell growth inhibition, apoptosis induction, and interference of cell cycle progression [34]. Suggested molecular targets for ASX-induced cancer prevention and treatment include NF-κB, Janus kinase (JAK)/STAT-3, phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt), MAPK, Nrf2, and PPARy [34]. Although some PPARy agonists have shown pro-tumorigenic activities [67–70], PPARy activation is also considered a promising therapeutic target for novel anticancer agents. This is based on its suppression of cellular growth and proliferation as well as its promotion of terminal differentiation and apoptosis [71–73].

As mentioned previously, a specific PPARy antagonist (GW9662) suppressed ASX-mediated induction of the connexin 43 gene in mouse embryonic fibroblasts (C3H/10T1/2) [45]. This result implies that cancer-preventive upregulation of connexin 43 is associated with ASX and its ability to activate PPARy. Zhang et al. [47] also demonstrated that ASX, in a time- and/or dose-dependent manner, inhibited cell growth, decreased cell viability, and induced cell cycle arrest and apoptosis in K562 leukemia cells, which are partly attenuated through the preincubation of GW9662.

In addition to PPARy, the roles of other PPAR subtypes in carcinogenesis and chemoprevention have been demonstrated [4]. For example, PPARα-dependent hepatocarcinogenesis has been reported in chronic rodent models [74], and PPARα agonists have been found to attenuate cell growth and angiogenesis in various tumor strains including A549 human non-small cell lung cancer, B16-F10 murine melanoma, Lewis lung carcinoma, U87 human glioblastoma, and HT-1080 human fibrosarcoma [75,76]. There is limited proof that downregulation of PPARβ/δ by several antagonists can inhibit tumorigenesis; thus, there are still conflicting opinions on the relationship between PPARβ/δ and cancer development, treatment, and prevention [77,78]. Further comprehensive research on ASX-mediated PPARα or PPARβ/δ modulation and therapeutic impacts on cancer progression are necessary to elucidate its possible anticancer properties.

3.3. Effects on Lipid and Glucose Metabolism

All PPAR subtypes are involved in lipid and carbohydrate metabolism as well as the management of metabolic syndrome and related disorders such as obesity, type 2 diabetes, atherosclerosis, and non-alcoholic fatty liver disease (NAFLD) [11,79]. Most studies have demonstrated that the physiological role of ASX is mainly focused on hepatic lipid and glucose metabolism via the modulation of PPARα and/or PPARy.

With its dual role as a PPARα agonist and PPARy antagonist, Jia et al. [39] showed that ASX treatment decreases intracellular cholesterol and triglyceride contents in lipid-loaded HepG2 cells. Furthermore, ASX altered the expression of genes that target PPARα and PPARy. It increased the expression of sterol carrier protein 2 (SCP2), acyl-CoA dehydrogenase very long chain (ACADVL), acyl-CoA dehydrogenase medium chain (ACADM), enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydroganase (EHHADH), and sterol 27-hydroxylase (CYP27A1). It also decreased the expression of carnitine palmitoyltransferase (CPT) 2 and aconitase 1 (ACO1), which are involved in various lipid and glucose metabolism pathways. The results from hepatic transcriptome profile analyses were comparable to those of the commonly used hypolipidemic agents fenofibrate and lovastatin, suggesting a preventive role for ASX in metabolic disorders associated with hepatic hyperlipidemia like NAFLD or NASH. The in vivo effects of ASX on lipid metabolism were also assessed by the same laboratory [40], and they observed significantly increased PPARα and reduced PPARy gene and protein expression in high-fat diet-fed mice with a co-treatment of ASX. Several PPARα and PPARy-related target genes that play key roles in fatty acid uptake (caveolin-1), fatty acid β-oxidation (CPT1 and acyl-CoA oxidase (ACOX) 1), triglyceride hydrolysis (lipoprotein lipase (LPL)), and mitochondrial thermogenesis (uncoupling protein 2 (UCP2)) were also upregulated by ASX administration. The expression of lipogenic genes regulated by PPARα such as sterol regulatory element binding protein (SREBP) 1c, fatty acid synthase (FAS), and acetyl-CoA carboxylate 1 (ACC1) were not affected by ASX; however,
LXRα, which may contribute to an increase in plasma high-density lipoprotein (HDL) cholesterol levels, was affected by ASX. In addition, ASX reduced hepatic steatosis through PPAR-mediated inhibition of the Akt-mTOR axis and activation of autophagy pathways. The authors concluded that ASX treatment ameliorates high-fat diet-induced hepatic lipid accumulation and hepatic steatosis in mice via differential regulatory actions involving PPARα and PPARγ. Another in vivo study by Xu et al. [41] investigated the effect of an ASX and flaxseed oil combination on hepatic lipid accumulation and oxidative stress. The combination resulted in the upregulation of PPARα protein expression and the increased mRNA expression of CPT1 and ACOX in high-fat diet-fed male rats. It also caused the downregulation of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and SREBP1 proteins and decreased mRNA levels for FAS and ACC. These results could support the hepatoprotective properties of ASX and flaxseed oil confirmed by the reversal of hepatic steatosis, decrease in hepatic triglyceride and total cholesterol levels, and enhancement in liver antioxidant capacity.

In contrast, increased PPARγ expression was associated with the development of hepatic steatosis and lipotoxicity in GY zebrafish [50]. Overexpression of YY1 transcription factor caused yellow and greasy liver appearance as well as marked lipid accumulation in hepatocytes compared to control zebrafish lines. The mRNA expression levels were also increased in genes responsible for fatty acid synthesis, transport and binding, lipid storage, and hepatic lipogenesis. YY1-mediated suppression of CHOP-10 expression caused upregulation of C/EBPα and PPARγ and related target genes such as adipocyte protein 2 (aP2), caveolin-1, adiponectin, adipsin, and fat-specific gene 27 (FSP27). As a result of progressive hepatic steatosis, more than 90% of adult GY fish exhibited liver abnormalities (i.e., gross liver hypoplasia) and related lipotoxicity, which included increased lipid peroxidation, ROS generation, gene expression involving lipid oxidation, and lipo-apoptosis.

Kobori et al. [42] presented different results from those of previous studies and in which ASX was predicted to decrease the actions of PPARα and PPARβ/δ and reduce the mRNA levels of related genes in mice with diet-induced NASH. In addition, the expressions of PPAR target molecules such as patatin-like phospholipase domain containing 2 (PNPLA2) and promyelocytic leukemia protein (PML) were also affected by ASX. The authors suggested that the inhibitory effect of ASX on hepatic gene expression, which leads to reduced mitochondrial fatty acid oxidation, is attributable to the suppression of PPARα activity. Thus, further scientific studies are required to elucidate the molecular actions of ASX on PPAR function.

Several studies have also shown the effect of ASX on lipid and/or glucose control using other cellular resources. ASX completely inhibited rosiglitazone-induced lipid accumulation and reduced the mRNA expression of PPARγ target genes (aP2, fatty acid-binding protein, and LPL) in 3T3-L1 adipocyte [43]. In contrast, enhanced adipogenic differentiation and significant overexpression of PPARγ and other adipogenic genes were observed in ASX-treated neural stem cells [49]. More recently, Rundblad et al. [44] reported that the intake of ASX-added HOSO supplementation downregulates the expression of PPARβ/δ, as well as other genes affecting lipid and glucose metabolism, in PBMCs from healthy subjects.

4. Conclusions and Future Prospects

ASX exhibits significant anti-inflammatory and anticancer properties, and it helps regulate lipid and glucose metabolism based on its differential modulation of PPARs depending on the type of cells. Like other previously reported molecular mechanisms, ASX-mediated PPARγ activation induces the expression of many pro-inflammatory molecules in macrophages and gastric epithelial cells. Despite the conflicting views regarding the effect of PPARs on cancer, the agonistic effect on PPARγ by ASX treatment leads to the inhibition of growth and cell cycle as well as an induction of apoptosis against several tumor cells. The effects of ASX on lipid and glucose metabolism associated with PPAR modulation are somewhat complicated. Simultaneous PPARα activation and PPARγ suppression play a major role in hepatic lipid homeostasis as well as NAFLD and NASH, but the overexpression of
PPARγ causes excessive hepatic lipid accumulation. Changes in PPAR expression also affect lipid and glucose metabolism in adipocytes and PBMCs.

PPARs are distributed ubiquitously and are involved in maintaining homeostasis and controlling diseases in the human body. A variety of PPAR ligands have been discovered, and though they are known to play a role in metabolic disorders such as type 2 diabetes and dyslipidemia, research on their new therapeutic potential is ongoing because of their vast influence on human health. For example, PPARγ has been found to provide additional benefits for cardiovascular homeostasis and related functional problems including atherosclerosis, restenosis, and hypertension [80]. PPARγ also plays a beneficial role in neurodegenerative disorders such as Parkinsonism, amyotrophic lateral sclerosis, Alzheimer’s disease and brain injury, and ocular diseases [6,81,82]. Furthermore, PPARβ/δ has been implicated in cardiac function, epidermal biology, neuroprotection, and gastrointestinal tract functions [83–86]. Spurred by these findings, numerous clinical trials are underway, which are exploring PPAR-based therapies for the treatment of many diseases [87].

Considering that ASX has a wide range of biological activities not covered in detail in this review, including anti-gastric, cardioprotective, immuno-modulatory, neuroprotective, retinoprotective, and osteogenic effects [33,37], further comprehensive studies of ASX-mediated influence on PPAR activity and its therapeutic outcomes in various pathophysiological conditions are necessary to clarify the role of ASX as a novel nutraceutical PPAR modulator.

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Antioxidant properties evaluation of topical astaxanthin formulations as anti-aging products

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Summary

Background: The reactive oxygen species lead to skin aging via oxidative damage that are induced by UV radiation. Therefore, topical formulations which have antioxidant effect could reduce aging level. Astaxanthin is an antioxidant substance.

Aims: The aim of this study was to investigate antioxidant activity and cytotoxicity potential of the astaxanthin-loaded gel formulations.

Methods: Astaxanthin-loaded oleoresin and algae extract were used as natural active materials. The lipogel and hydrogel of these natural materials were prepared as anti-aging formulations. The formulations were characterized via parameters such as, pH, rheological analysis, mechanical properties, and stability. And also in vitro release experiments of the formulations were carried out. The antioxidant activity and cytotoxicity test were performed.

Results: The results of characterization studies confirmed the formulations suitable for topical application. After 24 hours, 99 μg, 88.3 μg, 403 μg, and 234.8 μg of astaxanthin released through oleoresin lipogel, oleoresin hydrogel, algae extract lipogel, and algae extract hydrogel, respectively. It was found by the cytotoxicity tests that astaxanthin is more proliferative in lipogel formulations compared to hydrogel formulations. And finally, the highest antioxidant activity was found in the algae extract hydrogel and algae extract lipogel formulation, respectively (P < .05).

Conclusions: Topical formulations of astaxanthin-loaded oleoresin and algae extract were prepared successfully. At the same time, according to antioxidant activity and release studies, algae extract loaded could be suggested as topical anti-aging formulations.

KEYWORDS

algae extract, anti-aging, antioxidant, astaxanthin, cell culture, gel systems

INTRODUCTION

Intrinsic factors such as genetics, cellular metabolism, hormone, and metabolic processes and extrinsic ones such as chronic light exposure, pollution, ionizing radiation, chemicals, and toxins cause the skin aging. Aging occurs through two different biological mechanisms which are chronological aging and photoaging and results functional and aesthetic skin changes.

The production of reactive oxygen species is induced via UV radiation. The reactive oxygen species lead to skin aging by oxidative products and oxidative damage, which are the markers of oxidative stress. The antioxidant defense is one of the effective skin...
defense mechanisms. Enzymes and antioxidant substances react directly with reactive oxygen species and preventing them from reaching their biological target.\(^4,5\) UV radiation is an external factor which influences the photoaging. It can be prevented by various antioxidants in a variety of skincare products.

In this study, astaxanthin (3,3’P-dihydroxyl-4,4’P-dioxo-L-carotene) was chosen as an effective antioxidant agent. It is a common lipophilic pigment found in algae, fish, and birds.\(^6,7\) It is more efficient than many antioxidant agents such as carotenoid and vitamin E. Due to antioxidant effect, it could be used as anti-aging agent caused by UV radiation. Although there are few studies that mentioned topical preparation of astaxanthin,\(^8-11\) there is no any commercial product.

Gel formulations have many advantages such as low cost and ease of use, production and scale-up. In this study, astaxanthin-containing oleoresin and algae extract were used as antioxidant materials to prepare hydrogel and lipogel formulations as anti-aging products. Gel formulations containing astaxanthin were characterized via parameters, pH, rheological and mechanical properties, and stability. In vitro release experiments of the formulations, antioxidant activity assay for the determination of efficiency and cell culture studies for cytotoxicity test were performed.

### TABLE 1  The compositions of prepared gel formulations

| Codes  | Carbopol 974P | Distilled water | Lecithin | Isopropyl myristate | Propylene glycol | Ethanol | AE | AO |
|--------|--------------|----------------|----------|---------------------|------------------|--------|----|----|
| AE-L   | 0.96         | 46.6           | 0.24     | 4.8                 | 27.6             | 13.5   | 6.3|    |
| AO-L   | 1            | 49             | 0.25     | 5                   | 29               | 14.75  | 1  |    |
| AE-K   | 2            | 91.7           | 6.3      |                     |                  |        |    |    |
| AO-K   | 2            | 97             | 1        |                     |                  |        |    |    |

AE: astaxanthin algae extract, AO: astaxanthin oleoresin, AE-L: lipogel of algae extract, AO-L: lipogel of oleoresin, AE-K: hydrogel of algae extract, AO-K: hydrogel of oleoresin.

Then, astaxanthin oleoresin or astaxanthin algae extract was added to mixture (phase A). Carbopol was mixed with distilled water and kept at room temperature over the night for swelling. Triethanolamine was added for neutralization and gelling of polymer (phase B). Finally, astaxanthin mixture (phase A) was added to carbopol gel system (phase B) to obtain astaxanthin lipogel formulations (AO-L and AE-L),

Carbopol 974P gel systems were used to obtain hydrogel formulations of astaxanthin. Hydrogel systems were prepared as mentioned above (phase B), Astaxanthin oleoresin (AO-H) and astaxanthin algae extract (AE-H) were added to gels and mixed by magnetic stirrer. The astaxanthin concentration of all formulations was 500 μg/g (Table 1).

### 2.3  Characterization of formulations

The macroscopical analysis of formulations was performed via controlling appearance, phase separation, and color of formulations.

The pH values of the formulations were determined using a pH meter (Jenway 3040 Ion Analyze) at 25 ± 1°C. All experiments were replicated at least three times.

The rheological analysis of the formulations was performed at 32 ± 0.1°C and at 25 ± 0.1°C using an AR 2000 controlled stress/controlled rate rheometer (Haake MARS, Platte PP35 Ti, plate cover MPC35, Karlsruhe, Germany). In continuous shear analysis, the upward and downward flow curves for each formulation were measured over shear rates ranging from 10 to 900 s\(^{-1}\). All experiments were replicated at least five times.

Oscillatory analysis of each formulation was performed at 25 ± 0.1°C and 32 ± 0.1°C, where stress was directly proportional to strain and the storage modulus remained constant. Frequency sweep analysis was carried out over the frequency range of 0.1-10.0 Hz following application of a constant stress. Storage modulus (G\(^*\)) and loss modulus (G\(^"\)), the dynamic viscosity (η\(^d\)), and the loss tangent (tan \(\delta\)) were determined as previously described by the authors.\(^14,15\)

The mechanical behavior of the formulations was measured using a TA-XT Plus Texture Analyzer ( Stable Micro Systems UK) equipped with 5-kg load cell in texture profile analysis (TPA) mode. From the resultant force-time curve, the mechanical parameters (hardness, compressibility, adhesiveness, and cohesiveness) were determined.\(^16\) Experiments were carried out at least five times, and standard deviations were calculated.

The stability of formulations was studied for 3 months by the means of drug content, pH, and viscosity. The formulations were...
kept in stability cabinet (Nüve TK 252, Turkey) 25 ± 0.1°C and 60% relative humidity and in refrigerator +4 ± 0.1°C.

2.4 Analytical method for determination of astaxanthin

The HPLC system is consisting of a DAD detector and a C18 column (25cm × 4.6 mm). A filtered and degassed solution containing acetonitrile:water:methanol:dichloromethane (45.5:4.5:28:22) was used as the mobile phase. The flow rate was 1 mL/min. The wave legend was set at 480 nm. The peak area correlated linearly with astaxanthin concentrations in the range of 0.1-25 μg/mL (r² = 0.999). The validation of the method was accomplished on the specificity, stability, the accuracy, and the precision.17

2.5 Determination of astaxanthin in algae extract

The weighted algae extract was mixed with DMSO completely. The clear solutions were filtrated through a 0.45-μm pore size membrane filter, and the concentrations of active agent were found with validated HPLC method. The study was replicated five times.

2.6 Solubility study of astaxanthin algae extract

The algae extract was added to with phosphate-buffered saline (PBS): ethanol (3:7, v/v) mixture and then mixed with horizontal mixture for 24 hours. After filtration by membrane filter (0.45 μm), the concentration of astaxanthin in solution was determined by validated HPLC method.

2.7 In vitro drug release

The concentration of astaxanthin in the gels was determined before starting in vitro release studies. The certain amount of gel formulations was dissolved with DMSO and mixed with vortex for 15 minutes. After filtration, the active agent concentrations were found. The study was performed with formulations without drug to show specificity of analytical method.

The dialysis bag diffusion technique was used to study the in vitro drug release of astaxanthin. 1 g of lipogel and hydrogel of formulations was placed in the dialysis bag (cellulose membrane, molecular weight cutoff 12-14 000 Da), hermetically sealed, and immersed into 100 mL of PBS (pH 7.4); ethanol (7:3, v/v) mixture. The entire system was kept at 37 ± 0.5°C with continuous magnetic stirring at 600 rpm/min. Samples were withdrawn from the receptor compartment at predetermined time intervals and replaced by fresh medium. The amount of drug released was determined by HPLC.

2.8 Cell culture studies

Cytotoxicity potential of the formulations was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay.18 MTT reagent is a yellow substrate which produces a dark blue formazan product when incubated with viable cells. Therefore, the level of the reduction in MTT to form formazan can reflect the level of cell metabolism.

Human kidney proximal tubular epithelial cell line (HK-2, ATCC) was cultured in 10% fetal bovine serum-supplemented DMEM: F12 medium. Cells were plated in 96-well plates at a density of 5 × 10³ cells/well and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 24 hours for cell attachment. Then, the cells were treated with 2 nmol/L, 4 nmol/L, 8 nmol/L, 16 nmol/L ve 32 nmol/L of the astaxanthin formulations and cells were incubated for 48 hour. DMSO was used as a control group. At the end of the incubation time, the medium of each cell culture was replaced by 1 mL of complete medium, to which 0.01 mL of the MTT stock solution (5 mg/mL) was added. Cells were incubated for 4 hour at 37°C for, after which the medium was removed and the culture washed with PBS. 0.01 mL DMSO was added to dissolve the formazan crystals then transferred to a 96-well plate. The absorbance of the formazan solution was measured in a plate reader (VersaMax) at 540 nm. The ratio of the absorbance of treated samples to the absorbance control (taken as 100%) was expressed as % cell viability. Cell survival was expressed as the percentage of formazan absorbance. Results were the mean ± standard deviation (mean ± SD) from at least three different experiments in triplicate.

2.9 Antioxidant activity studies

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and Trolox antioxidant equivalent capacity (TEAC) methods were used for antioxidant activity of astaxanthin.

1,1-Diphenyl-2-picrylhydrazyl radical scavenging activity was tested according to the methods described previously.19 Ascorbic acid was used as a standard. Different concentrations of formulation samples (2, 4, 8, 12, 16, 20 μg/mL) and standards were prepared in DMSO. In a 96-well plate, 100 μL of DPPH (200 μmol/L) was mixed with 100 μL of samples and standards. The mixtures were incubated at room temperature for 30 minutes. The UV/Vis absorbance of the residual DPPH solution was determined at 517 nm in a microplate reader. The inhibition percentage was calculated following equation. (Ab is the absorbance of the blank, and Aa is the absorbance of the sample).

\[
\text{Inhibition } \% = \left( \frac{\text{Ab} - \text{Aa}}{\text{Ab}} \right) \times 100
\]

Trolox equivalent antioxidant capacity (TEAC) of formulations was determined by an improved assay based on the decolorization of the radical monocation of [2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS⁺)20 with minor modifications, applied to a 96-well microplate assay.21 Trolox was used as an antioxidant standard. 2.45 mmol/L potassium persulfate was mixed with 7 mmol/L ABTS, to produce an ABTS⁺ radical. This radical was left in a dark room at room temperature for 16 hours to reach stable absorbance at 734 nm.

Formulations were solved in DMSO to a final concentration 1 mg/1 mL. 1 mL 5 mmol/L phosphate buffer (pH: 7.4) was used as
a blank. The ABTS⁺ radical was diluted in 5 mmol/L phosphate buffer until it had an absorbance of 0.70 ± 0.02 at 734 nm. Next, 200 μL of this diluted solution was taken and mixed with 2 μL of various concentrations of formulations (1-1000 μmol/L). The optical density was determined spectrophotometrically at 734 nm at 30°C in a microplate reader (Thermo Varioskan Flash) for 1-6 minutes after the initial mixing. All measurements were carried out in triplicate. The inhibition percentages were calculated as follows, where $A_{\text{ABTS}^+}$ is the absorbance of the ABTS⁺ at 734 nm and $A_{\text{Amin}}$ is the absorbance after the addition of the sample to the ABTS⁺.

$$\text{Inhibition} \% = \left(\frac{A_{\text{ABTS}^+} - A_{\text{Amin}}}{A_{\text{ABTS}^+}}\right) \times 100$$ (2)

The standard curve of the Trolox was obtained using a Trolox standard solution at various concentrations (0.25-2.5 mmol/L) in 5 mmol/L phosphate buffer (pH: 7.4). The absorbance of the samples was compared to that of the standard curve, and the antioxidant properties were expressed as mmol/L Trolox equivalent.

### 2.10 The limitations of the study

The study has some limitations. Firstly, the in vivo studies could not perform because the ethic committee does not allow animal use for cosmetic products. Secondly, the ex vivo studies were not performed, because the prepared formulations would be used topically. The authors do not expect any systemic effect.

### 2.11 Statistical analysis

Data were expressed as mean ± standard deviations (mean ± SD). Differences among the groups were analyzed using one-way ANOVA followed, where appropriate, by Tukey’s post hoc test. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software). For all comparisons, a value of $P < .05$ was considered significant. Results were derived from three independent experiments.

### 3 RESULTS AND DISCUSSION

#### 3.1 Characterization of formulations

Oleoresin formulations had purple color, and algae extract formulations had red color. Lipogel formulations had less viscosity than hydrogel due to lower concentration of polymer. The pH of formulations was range from 4.5 to 5. The pH values are suitable for topical application as the skin pH is 5.5.22 The results of macroscopic observation and pH are shown in Table 2.

The rheological properties of gel systems affect both the ease of application and spreadability on skin. Representative flow curves were graphically presented in Figure 1. The shear stress changes upon shear rates have been used to determine whether the rheological behavior of the formulation is Newtonian or non-Newtonian. The shear stress is increasing with increasing shear rate. The flow is not starting from origin point, and this showed the formulations exhibited plastic flow at 25°C and at 32°C. As shown in Figure 1, viscosity decreases with increasing shear rate, as expected from plastic fluids. The lipogel formulations showed lower viscosity than hydrogels. Lipogel formulations which were prepared with oleoresin astaxanthin had higher viscosity than algae extract astaxanthin due to viscosity of resin.

The oscillatory stresses on the viscoelastic properties are measured from two dynamic moduli: One is the storage modulus, $G'$, and other is the loss modulus, $G''$. $G'$ measures the elasticity of system, and $G''$ represents viscous components. A gel system exhibits a solid-like mechanical spectrum, that is, $G' > G''$.15

Tables 3 and 4 represent the frequency dependence of the $G'$ and $G''$ of gel formulations at 25°C and 32°C. As shown, all formulations found were nearly frequency independent after certain frequency values and they exhibited typical gel-type mechanical spectra ($G' > G''$) at 32°C. AO-L and AO-K formulations could be described as strong cross-linked gel, and they did not influence by the frequency of oscillation.

The value of the loss tangent (tan δ = $G''/G'$) is a measure of the relative contribution of viscous components to the mechanical properties of the materials. For solid systems, it should be <1, and for liquid systems, it should be >1.23 For all gel formulations, the loss tangent was found <1 for both degrees (Table 5).

The textural properties of gels were performed to assess the applicability of gels to skin (Table 6). Mechanical parameters such as hardness, compressibility, adhesiveness, cohesiveness, and elasticity were defined from the resultant force-time curve.12 The gel systems could be easily removed from container and spread on skin surface when the gel hardness and compressibility are low.15 Cohesiveness and adhesiveness are important parameters for the structural properties of formulations. A lower value of elasticity indicates higher product elasticity, and this shows product could be easily transformed from deformed to undeformed conditions. The results show that carbopol affects the structural properties of formulations. Formulations jellified with carbopol had similar hardness and other textural values. Lipogel prepared with algae extract had lowest values. On the other hand, oleoresin lipogel showed higher textural values than lipogel prepared with algae extract due to mechanical properties of oleoresin.

The result of stability studies showed that the formulations were stable during 3 months under mentioned conditions. The pH and viscosity of formulations did not change after 3 months. Table 7

### Table 2: Macroscopic observation and pH of gel systems

| Code   | Macroscopic observation          | pH  |
|--------|----------------------------------|-----|
| AO-L   | Purple color, homogeneous, fluidic| 4.75|
| AO-K   | Purple color, homogeneous, viscose| 4.66|
| AE-L   | Red color, homogeneous, fluidic  | 4.87|
| AE-K   | Red color, homogenous, viscose   | 4.76|
| Lipogel| White color, homogenous, fluidic | 4.64|
| Hydrogel| Transparent, homogeneous, viscose| 4.52|

AE-L: lipogel of algae extract, AO-L: lipogel of oleoresin, AE-K: hydrogel of algae extract, AO-K: hydrogel of oleoresin.
indicates the percentage of drug content in the formulations. As shown, a slight change was observed for both of the conditions ($P > .05$).

### 3.2 Analytical method for determination of astaxanthin

A validated HPLC method was developed to determine the astaxanthin in samples. The variation coefficient of accuracy, precision, and solution stability found was lesser than two percent, indicating the method was validated. The specificity of method was proved by injecting the formulations without drug to HPLC system. The limit of detection (LOD) and limit of quantification (LOQ) were also determined, and the results found were $3.3 \times 10^{-4} \text{ g/mL}$ and $10^{-3} \text{ g/mL}$, respectively.

### 3.3 Determination of astaxanthin in algae extract

The drug concentration of algae extract was not known. The amount of drug in oleoresin was determined by company. The drug content in algae extract was determined by the use of oleoresin as standard. And it was found that the astaxanthin concentration in extract is 0.8%.

The solubility of algae extract in PBS:ethanol (3:7, v/v) mixture found was $56 \pm 2.1 \text{ g/mL}$.
**TABLE 4** Effect of temperature on the loss modulus (\(G''\)) of formulations at five representative frequencies

| Codes | \(\theta^\circ\) | 0.60 Hz | 2.00 Hz | 5.00 Hz | 7.00 Hz | 10.00 Hz |
|-------|----------------|---------|---------|---------|---------|----------|
| AE-K  | 25             | 22.57 \ ± 0.03 | 31.04 \ ± 0.03 | 29.04 \ ± 0.03 | 31.04 \ ± 0.03 | 29.04 \ ± 0.03 |
|        | 32             | 22.57 \ ± 0.03 | 31.04 \ ± 0.03 | 29.04 \ ± 0.03 | 31.04 \ ± 0.03 | 29.04 \ ± 0.03 |
| AE-L  | 25             | 6.57 \ ± 0.05 | 9.57 \ ± 0.05 | 12.57 \ ± 0.05 | 15.57 \ ± 0.05 | 18.57 \ ± 0.05 |
|        | 32             | 6.57 \ ± 0.05 | 9.57 \ ± 0.05 | 12.57 \ ± 0.05 | 15.57 \ ± 0.05 | 18.57 \ ± 0.05 |
| AO-K  | 25             | 24.57 \ ± 0.05 | 35.47 \ ± 0.05 | 28.47 \ ± 0.05 | 21.47 \ ± 0.05 | 14.47 \ ± 0.05 |
|        | 32             | 24.57 \ ± 0.05 | 35.47 \ ± 0.05 | 28.47 \ ± 0.05 | 21.47 \ ± 0.05 | 14.47 \ ± 0.05 |
| AO-L  | 25             | 17.86 \ ± 0.08 | 27.86 \ ± 0.08 | 17.86 \ ± 0.08 | 17.86 \ ± 0.08 | 17.86 \ ± 0.08 |
|        | 32             | 17.86 \ ± 0.08 | 27.86 \ ± 0.08 | 17.86 \ ± 0.08 | 17.86 \ ± 0.08 | 17.86 \ ± 0.08 |

AE-L: lipogel of algae extract, AO-L: lipogel of oleoresin, AE-K: hydrogel of algae extract, AO-K: hydrogel of oleoresin.

**TABLE 5** Effect of temperature on the loss tangent (\(\tan \delta\)) of formulations at five representative frequencies

| Codes | \(\theta^\circ\) | 0.60 Hz | 2.00 Hz | 5.00 Hz | 7.00 Hz | 10.00 Hz |
|-------|----------------|---------|---------|---------|---------|----------|
| AE-K  | 25             | 0.079 \ ± 0.02 | 0.098 \ ± 0.02 | 0.128 \ ± 0.02 | 0.138 \ ± 0.02 | 0.159 \ ± 0.03 |
|        | 32             | 0.079 \ ± 0.02 | 0.098 \ ± 0.02 | 0.128 \ ± 0.02 | 0.138 \ ± 0.02 | 0.159 \ ± 0.03 |
| AE-L  | 25             | 0.195 \ ± 0.01 | 0.255 \ ± 0.01 | 0.333 \ ± 0.01 | 0.406 \ ± 0.01 | 0.412 \ ± 0.03 |
|        | 32             | 0.195 \ ± 0.01 | 0.255 \ ± 0.01 | 0.333 \ ± 0.01 | 0.406 \ ± 0.01 | 0.412 \ ± 0.03 |
| AO-K  | 25             | 0.067 \ ± 0.02 | 0.074 \ ± 0.02 | 0.090 \ ± 0.02 | 0.094 \ ± 0.02 | 0.111 \ ± 0.02 |
|        | 32             | 0.067 \ ± 0.02 | 0.074 \ ± 0.02 | 0.090 \ ± 0.02 | 0.094 \ ± 0.02 | 0.111 \ ± 0.02 |
| AO-L  | 25             | 0.057 \ ± 0.01 | 0.062 \ ± 0.01 | 0.082 \ ± 0.01 | 0.088 \ ± 0.01 | 0.103 \ ± 0.02 |
|        | 32             | 0.057 \ ± 0.01 | 0.062 \ ± 0.01 | 0.082 \ ± 0.01 | 0.088 \ ± 0.01 | 0.103 \ ± 0.02 |

AE-L: lipogel of algae extract, AO-L: lipogel of oleoresin, AE-K: hydrogel of algae extract, AO-K: hydrogel of oleoresin.

**TABLE 6** Mechanical properties of gel formulations

| Codes | Hardness (mN ± SS) | Adhesiveness (mN.mm ± SS) | Cohesiveness (mN.mm ± SS) | Compressibility (mN.mm ± SS) | Elasticity (mN.mm ± SS) |
|-------|-------------------|---------------------------|--------------------------|-----------------------------|------------------------|
| AO-L  | 119.92 \ ± 0.002 | 331.28 \ ± 0.002 | 1.11 \ ± 0.003 | 517.63 \ ± 0.002 | 1.07 \ ± 0.001 |
| AO-K  | 124.63 \ ± 0.003 | 488.48 \ ± 0.001 | 1.07 \ ± 0.005 | 612.69 \ ± 0.002 | 0.99 \ ± 0.002 |
| AE-L  | 23.69 \ ± 0.001 | 32.75 \ ± 0.002 | 1.19 \ ± 0.002 | 93.00 \ ± 0.004 | 0.95 \ ± 0.001 |
| AE-K  | 118.45 \ ± 0.001 | 341.52 \ ± 0.002 | 1.24 \ ± 0.004 | 572.56 \ ± 0.003 | 0.94 \ ± 0.001 |

AE-L: lipogel of algae extract, AO-L: lipogel of oleoresin, AE-K: hydrogel of algae extract, AO-K: hydrogel of oleoresin.

**TABLE 7** The percentages of drug in the formulations during stability study

| Codes | \(25 \pm 0.1^\circ\C/60\%\ RH\) | \(+4 \pm 0.1^\circ\C\) |
|-------|-------------------------------|------------------------|
|       | 1 mo                          | 2 mo                   | 3 mo                   |
|       | 1 mo                          | 2 mo                   | 3 mo                   |
| AE-L  | 98.7 \ ± 0.3%                | 98.5 \ ± 0.4%          | 98.4 \ ± 0.2%          | 99.8 \ ± 0.2%          | 99.6 \ ± 0.4%          | 99.5 \ ± 0.3%          |
| AE-K  | 98.5 \ ± 0.6%                | 98.3 \ ± 0.5%          | 98.1 \ ± 0.5%          | 99.8 \ ± 0.4%          | 99.4 \ ± 0.1%          | 99.1 \ ± 0.5%          |
| AO-L  | 98.4 \ ± 0.2%                | 98.4 \ ± 0.2%          | 98.3 \ ± 0.4%          | 99.5 \ ± 0.6%          | 99.3 \ ± 0.6%          | 99.3 \ ± 0.4%          |
| AO-K  | 98.2 \ ± 0.6%                | 98.2 \ ± 0.3%          | 98.1 \ ± 0.2%          | 99.7 \ ± 0.3%          | 99.6 \ ± 0.5%          | 99.4 \ ± 0.2%          |

AE-L: lipogel of algae extract, AO-L: lipogel of oleoresin, AE-K: hydrogel of algae extract, AO-K: hydrogel of oleoresin.

**3.4 | In Vitro release studies**

Figure 2 illustrates the released amount of astaxanthin from formulations. As shown, the mechanical properties and viscosity of formulations affect the release profile of active agents in gel systems. AE-L formulation had lower values in hardness and viscosity and as a result, the release percentage was higher. Oleoresin gel formulations had slower release due to resin content in the gels. The release from oleoresin hydrogel and lipogel found was nearly the same. After 24 hours, 99 \(\mu g\), 88.3 \(\mu g\), 403 \(\mu g\), and 234.8 \(\mu g\)
3.5 | Cell culture studies

To investigate the effects of AO, AE, and gel systems on cell proliferations, HK-2 cell was treated with formulation which contains different astaxanthin concentrations for 48 hours. MTT method was used to determine cell proliferation to compare the number of viable cell in the AO, AE, AO-L, AO-K, AE-L, and AE-K groups. Differences among the groups were analyzed using one-way analysis of variance (ANOVA) by GraphPad Prism 5.0 (GraphPad Software). For all comparisons, a value of $P < .05$ was considered significant. Results were derived from three independent experiments.

Figure 3 shows the results for control group and formulation. In previous studies, no negative effect was observed in terms of cell proliferation and viability ($P > .05$). Moreover, it was determined that AO and AE induced HK-2 cell proliferation in a dose-dependent manner.

The results of MTT assay showed that any of formulation had cytotoxic effects. Moreover, it was observed that AO and AO-L formulations caused a significant increase in cell proliferation compared to untreated and only vehicle-treated cells ($P < .01$), whereas there was no significant increase in AO-K formulation-treated cell ($P > .05$).

Similarly, it was determined that AE and AE-L caused a significant improvement in cell proliferation in a dose-dependent manner compared to untreated and only vehicle-treated cells ($P < .05$). On the other hand, there was no significant increase in AE-K formulation-treated cell ($P > .05$).

In previous studies, it has been demonstrated that astaxanthin improved epithelial cell proliferation due to antioxidant-dependent activity.\textsuperscript{24} Our findings indicated that astaxanthin also induced HK-2 cell proliferation in a dose-dependent manner. In addition, astaxanthin is more proliferative in lipogel formulation compared to hydrogel formulations.

3.6 | Antioxidant activity studies

The antioxidant activities of astaxanthin-containing hydrogel and lipogel formulations were conducted for two different radicals: DPPH and ABTS$^+$. The DPPH radical scavenging activity was presented as the percentage of radical scavenging ($\%$).

Figure 4 shows the $\%$ DPPH inhibition. Results indicated that there were no significant differences in the percentage of radicals scavenged in empty formulations of AO-K and AO-L ($P > .05$). The highest percentage of scavenged radicals was determined for an
addition of 0.5 mg/mL astaxanthin into AE-K (95.63 ± 4.2%) and AE-L (77.02 ± 4.3%) formulation \((P < .05)\), while the AO-L (29.04 ± 3.92%) and AO-L (18.5 ± 2.27%) formulations at different astaxanthin concentrations were slightly weaker scavengers in a dose-dependent manner \((P < .05)\). In addition, it was observed that AE showed significantly higher DPPH scavenging activity in K compared to L at 0.5 mg/mL concentration \((P < .05)\) while AO showed higher scavenging activity in L compared to K formulation \((P < .05)\).

Figure 5 shows the Trolox equivalent antioxidant capacities (mmol/L) of formulations. The ABTS⁺ radical scavenging activity of the formulation is measured relative to that of Trolox on a micromolar basis. Results showed that all formulations relatively scavenged the ABTS⁺ radical in a dose-dependent manner of astaxanthin. Similar to DPPH scavenging activity results, the highest antioxidant activity was determined for an addition of 0.5 mg/mL astaxanthin into AE-K and AE-L formulation \((P < .05)\). Moreover, it was determined that L formulation of AO and AE was showed significantly higher antioxidant activity compared to K at 0.5 mg/mL concentration \((P < .05)\).

However, it has been reported that astaxanthin is more effective than carotenoid and vitamin E, and there is a limited number of studies showing the antioxidant activity of astaxanthin in topical formulations.\(^{10,11}\) In this study, our results clearly indicate that the astaxanthin shows strong antioxidant activity in both lipogel and hydrogel formulations. These findings support previous studies about the antioxidant effects of astaxanthin in topical formulations.\(^{8-11}\) In addition, both DPPH and TEAC assays have shown that AE-containing formulations exhibit more effective antioxidant properties than
formulations containing AO, regardless of whether the formulation is lipogel- or hydrogel-forming.

4 | CONCLUSION

In this study, astaxanthin hydrogels and lipogels were prepared successfully for anti-aging. Astaxanthin algae extract lipogel formulation could be promising systems for topical application due to fast release profile and easier application. As a conclusion, an innovative topical formulation providing anti-aging efficacy, containing astaxanthin that had a strong antioxidant activity, was developed.

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CONFLICT OF INTEREST

The authors reveal no conflict of interest in this manuscript.

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Effects of Astaxanthin on Pulse Rate Variability of Mice Under Chronic Stress

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Abstract. This exploratory study aims to evaluate the autonomic cardiovascular effects of the astaxanthin on pulse rate variability (PRV) of chronically stressed mice.

We used male CD1 mice that were divided into four groups: I-control (Ct, n = 3), II-chronic restraint stress (St, n = 4), III-supplemented with astaxanthin (Astx, n = 4) and IV-supplemented and stressed (Astx + St, n = 5). Astaxanthin (4 mg/kg for 21 consecutive days) in the corresponding groups was orally administered. In the last day, the pulse rate signals were recorded by photoplethysmography. One minute of pulse-to-pulse time series (P-P) were constructed for all the groups. The P-P time series were processed to estimate relevant linear and nonlinear PRV parameters. It was found that in stressed mice, astaxanthin produced: (1) decreased heart rate; (2) increased complexity in the P-P time series and (3) increased fractal behavior in the pulse rate fluctuations. These results suggest that astaxanthin may attenuate the autonomic cardiac imbalance produced by chronic stress.

Keywords: Astaxanthin · Pulse rate variability · Autonomic nervous system · Chronic stress

1 Introduction

The ketocarotenoid astaxanthin has several applications in industries such as aquaculture, food, cosmetics, nutraceuticals, and pharmaceuticals. It is a micronutrient which is attributed numerous beneficial properties in health issues. The astaxanthin is considered the most potent antioxidant that exists in the natural environment. Furthermore, it is known that astaxanthin exerts, directly or indirectly, cardioprotective effects due to its anti-inflammatory, antihypertensive, antiobesogenic and lipid-lowering agent properties \cite{1, 7}. Interestingly, some studies have demonstrated that long-term astaxanthin administration to isoproterenol-induced rats produces significant cardioprotection by minimizing the alterations in the activities of the antioxidant enzymes. These effects could be due to membrane protective action of astaxanthin by scavenging the free radicals and its antioxidant action \cite{2}.

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Although psychological stress is considered a typical response to ensure survival and restore homeostasis, in a chronic way can lead to allostasis [3], which is related to metabolic imbalances (e.g., oxidative stress, dyslipidemia, hyperglycemia, changes in energy redistribution mediated by gluconeogenesis and glycogenolysis), and hemodynamic changes, which together can enhance cardiovascular risk [4].

It is well known that chronic stress causes autonomic imbalance, characterized by increased sympathetic activity and changes in parasympathetic activity [5]. Additionally, a noninvasive tool to assess the cardiac autonomic nervous system (ANS) is the analysis of pulse rate variability (PRV). Noteworthy, some authors have considered the PRV as a surrogate of heart rate variability (HRV) analysis [6]. Indeed, results speak in favor of enough accuracy of PRV to estimate HRV when subjects are at rest [7].

With this background, the present study aimed to evaluate the autonomic cardiovascular effects of the astaxanthin on pulse rate variability of chronically stressed mice. We hypothesize that cardioprotective effects of astaxanthin attenuate the cardiac autonomic imbalance produced by chronic stress in mice.

2 Materials and Methods

We used Male CD1 mice of 9–10 weeks old (30–5 g). Rodents were kept with water and food ad libitum under bioterium standard conditions (light/dark cycles 12/12 h, 20–24 °C, humidity 40–65%), and randomly allocated into four groups: I-control (Ct, n = 3), II-chronic restraint stress (St, n = 4), III-supplemented with astaxanthin (Astx, n = 4) and IV-supplemented and stressed (Astx + St, n = 5).

The St and Astx + St groups were stressed 3 h daily for 21 consecutive days, by confining them into rodent restrainers (2.36 in. long × 1.4 in. of diameter). Cyanotech Astaxanthin (2.5% microbeads presentation) was dissolved in water and tween-20% (5%), and it was orally administered (4 mg/kg) for 21 consecutive days in the corresponding groups. To avoid circadian cycle changes, the procedures were performed in a set schedule (8:00 a. m. ± 1 h), and to prevent adaptation to stress model; randomly stimuli were applied for 30 s every 20 min (restrainer’s inclination, rotation, and vibration). In the last day, continuous pulse rate signals were recorded at rest mice by a photoplethysmography system (BP-2000 Blood Pressure Analysis System, Visitech Systems, Inc., USA).

One minute of pulse-to-pulse time series (P-P) was calculated and filtered for all the groups using Matlab® software (the MathWorks, Inc. Natick, Massachusetts, USA). The P-P time series were processed to estimate relevant parameters such as the standard deviation of the PP intervals (SDPP), the heart rate (HR), the root mean square of the successive differences (RMSSD), the sample entropy (SampEn) and the short-term fractal scaling exponent (α1) for the four groups using Kubios software [8].

Ordinary one-way ANOVA was used to assess the effect of astaxanthin in the chronic stressed mice on linear PRV and SD measures. Normality was verified with the Shapiro-Wilk test. Data were further analyzed by Uncorrected Fisher’s LSD test for multiple comparisons. α = 0.05 was considered as the significance level. GraphPad software (Prism 7.0) was used to assess the statistical analyses. In all tests, the null hypothesis was rejected when P < 0.05.
3 Results

The results are presented as mean ± standard deviation (SD, Fig. 1). A significant decrement of SDPP was found in the St group in comparison with the Ct group (8.75 ± 2.66 vs 4.86 ± 0.78 ms, F_{St versus Ct} = 2.451, P < 0.05; Fig. 1a). Also, statistical differences were found between them for RMSSD (5.43 ± 2.68 vs 2.54 ± 0.67 ms, F_{St versus Ct} = 2.262, P < 0.05; Fig. 1c).

Fig. 1. Bar graphs of linear and nonlinear pulse rate variability (PRV) parameters of four groups of mice: control (Ct, n = 3), chronic restraint stress (St, n = 4), supplemented with astaxanthin (Astx, n = 4) and supplemented and stressed (Astx + St, n = 5): (a) the standard deviation of the PP intervals (SDPP); (b) the heart rate (HR); (c) the root mean square of the successive differences (RMSSD); (d) the sample entropy (SampEn) and (e) the short-term fractal scaling exponent (α1). *P < 0.05, **P<0.01 and ***P<0.001.
Furthermore, a significant decrement of HR is noted in the Astx + St group compared with St group (584.3 ± 14.9 vs 510.6 ± 8.49 bpm, F$_{St}$ versus Astx+St = 9.899, P < 0.001; Fig. 1b). The same parameter showed a considerable difference between Ct and Astx St (557.2 ± 28.42 vs 510.6 ± 8.49, F$_{Ct}$ versus Astx+ St = 9.899, P < 0.05; Fig. 1b).

Finally, the group St showed significant differences versus the Astx + St group for SampEn and for $\alpha_1$ (0.80 ± 0.26 vs 1.23 ± 0.12, F$_{Ct}$ versus Astx+ St = 5.826, P < 0.01; Fig. 1d) and (1.35 ± 0.11 vs 1.17 ± 0.06, Fig. 1e), respectively.

4 Discussion

At present, there are many advances in astaxanthin research related to cardiovascular health and diseases, due to its direct antioxidant, indirect antioxidant, anti-inflammatory, and anti-hypertensive properties [9]. On the other hand, it is well known that chronic stress is associated with a reduction of the autonomic modulation or sympathovagal balance modification and it is thereby considered by some authors to provoke cardiac autonomic dysregulation [10]. To our best knowledge, this is the first study documenting the effects of astaxanthin on the ANS. The key contribution of this work is to show that astaxanthin exert cardioprotective effects under chronic stress conditions as a result of enhanced autonomic cardiac modulation.

Recent studies have shown that astaxanthin supplementation improves parameters associated with brain health (neuro-inflammation and cognition), that data suggested that natural astaxanthin supplementation reduces negative mood state parameters (depression and fatigue) and improves global mood state and supports mental wellness [11]. Thus, we can speculate that astaxanthin is effective to attenuate stress. Additionally, other results suggest that astaxanthin can modulate the oxidative condition and may improve vascular elastin and arterial wall thickness in hypertension [12].

Our findings are in line with studies that indicate that supplementation with astaxanthin may modify autonomic function [13] and also protects the myocardium when is administered both orally or intravenously before the induction of the ischemic event [14]. On the one hand, relevant linear parameters such as SDPP and RMSSD shoed decreased values in chronically stressed mice group compared with a control group. These findings show that there is effectively a decrease in parasympathetic activity. Moreover, we observed a decreased values of HR in the astaxanthin supplemented mice, compared with both the Ct group and the St group, being this difference more noticeable in the group St. This findings may suggest that astaxanthin supplementation has a major effect under a stress condition. Finally, we found an increment of SampEn in the Astx + St group, it could be associated with more complexity in the time series. These indirect determinations are associated with compensatory response or adverse cardiovascular effects [15].

In future studies, we will verify the effects of astaxanthin on HRV and increase the number of subjects.
5 Conclusion

Chronic stress caused a decrease in PRV, accompanied by a decrease in parasympathetic cardiac activity. In addition, we found that in stressed mice, astaxanthin caused: (1) decreased heart rate; (2) increased complexity in the P-P time series and (3) increased fractal behavior in the pulse rate fluctuations. Concretely these results suggest that astaxanthin may exert potential cardioprotective effects under chronic stress conditions. Our results indicate that astaxanthin may attenuate the autonomic cardiac imbalance produced by chronic stress in mice increasing the parasympathetic tone.

Conflicts of Interest. All coauthors have no financial conflicts or other conflicts of interest in this area.

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The Neuroprotective Effects of Astaxanthin: Therapeutic Targets and Clinical Perspective

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Abstract: As the leading causes of human disability and mortality, neurological diseases affect millions of people worldwide and are on the rise. Although the general roles of several signaling pathways in the pathogenesis of neurodegenerative disorders have so far been identified, the exact pathophysiology of neuronal disorders and their effective treatments have not yet been precisely elucidated. This requires multi-target treatments, which should simultaneously attenuate neuronal inflammation, oxidative stress, and apoptosis. In this regard, astaxanthin (AST) has gained growing interest as a multi-target pharmacological agent against neurological disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD), brain and spinal cord injuries, neuropathic pain (NP), aging, depression, and autism. The present review highlights the neuroprotective effects of AST mainly based on its anti-inflammatory, antioxidative, and anti-apoptotic properties that underlies its pharmacological mechanisms of action to tackle neurodegeneration. The need to develop novel AST delivery systems, including nanoformulations, targeted therapy, and beyond, is also considered.

Keywords: neurodegenerative diseases; astaxanthin; pharmacology; neuroprotective agent; oxidative stress; neuroinflammation; apoptosis; drug delivery system

1. Introduction

Neurodegenerative disorder, as a common cause of human disability and death, is a term referring to progressive, symmetric, and selective loss of sensory, motor, and cognitive neuronal structure/function leading to neuronal cell death [1]. The death of neurons underlies the symptoms of several chronic or acute neurological disorders including Parkinson’s disease (PD), Alzheimer’s disease (AD), and brain or spinal cord injuries [2]. Neuronal cell death also affects depression, neuropathic pain (NP), aging, and autism as other neurological disorders [3].

Several causative factors are behind the etiology of neuronal disorders such as oxidative stress, inflammation, and apoptosis, as the main pathological pathways, and they play destructive roles in neuronal cell death and neurodegenerative processes [4]. Microglia activation and cytokines/chemokines release of the inflammatory pathways [5], as well as reactive oxygen species (ROS) and mitochondrial damages in the oxidative stress pathway [6], have destructive effects on neurodegenerative processes, which finally lead to cell death [4].
In spite of many developments in the field of clinical healthcare, neuroprotective therapies of neurodegeneration and neuronal death-related disorders have still remained as clinical challenges with no effective solution. Therefore, the need to develop novel multi-target therapeutics is felt to regulate more involving the signaling pathways and is thought to improve the life quality of individuals with neurodegenerative diseases.

Carotenoids are red-orange lipophilic pigments found in nature [7,8] with protective effects for human health. Thanks to their potential biological activities and health benefits, carotenoids have been receiving growing attention [4,9]. Several reports have attributed positive effects to their antioxidant activities [10,11]. Being the strongest antioxidant between the carotenoids [9], Astaxanthin (AST) is a lipid-soluble keto-carotenoid, belonging to xanthophylls, which has gained attention in experimental methods due to its neuroprotective features [12,13].

AST can be isolated mostly from microalgae Haematococcus pluvialis. However, shrimp, asteroidean, algae, lobster, crustacean, krill, trout, red sea bream, and salmon as marine animals and seafood are considered as other sources of extraction [4]. AST is chemically known as 3,3′-dihydroxy-β, β′-carotene-4,4′-dione (Figure 1), with the molecular formula of C_{40}H_{52}O_{4} [14]. It possesses a linear polar-nonpolar-polar structure with keto- and hydroxyl moieties at polar ends and conjugated carbon-carbon double bonds at a non-polar middle part, which allows it to fit specifically into the same span of cell membranes and pass through blood-brain barrier (BBB) [15].

![Figure 1. Chemical structure of Astaxanthin (AST).](image-url)

It has been shown that AST can block oxidative stress, inflammation, and apoptosis as the key pathways of neurodegeneration [4]. In the inflammatory pathway, AST blocks the macrophage migration inhibitory factor (MIF) as an up-stream cytokine, N-methyl-D-aspartate (NMDA) receptor 2B (NR2B) [12,13], and IkB kinase β (Ikk β). Thus, it inhibits the release of interleukins (ILs), tumor necrosis factor alpha (TNF-α), intercellular adhesion molecule 1 (ICAM1), and monocyte chemoattractant protein-1 (MCP-1) [4]. To tackle the oxidative stress, AST inhibits phosphorylated extracellular regulated protein kinase/extracellular regulated protein kinase ratio (p-ERK/ERK) [12,13], activates Nrf2/antioxidant response elements (Nrf2/ARE), and increases the release of heme oxygenase-1 (HO-1), glutathione S-transferase-α1 (GST-α1), and NAD(P)H quinine oxidoreductase-1 (NQO-1) [4,16]. Indeed, as the major signaling pathway against oxidative stress, Kelch-like ECH-associated protein 1 (Keap1)-Nrf2-ARE plays a key role in the cellular antioxidant response. In the non-stressed situation, Keap1 degrades the Nrf2 protein in the cytoplasm through the proteasome. Upon the oxidative stress, Nrf2 is rapidly degraded by proteasomes via the interaction with Keap1. This modification leads to the cytoplasmic accumulation and the nucleus translocation of newly synthesized Nrf2 in order to bind to the ARE [17,18]. Nrf2-ARE complex, in turn, attenuates the expression of NAD(P)H quinine oxidoreductase-1, superoxide dismutase (SOD), HO-1, and other regulatory enzymes to activate the defense system [18,19]. While glutathione reductase and thioredoxin reductase protect the complexes stability of Keap1-Nrf2 as well as IκB-NF-κB, cytosolic H_{2}O_{2} cause dissociation of the complexes and allows the nuclear transportation of NF-κB and Nrf2 [20,21]. In this line, the Nrf2-ARE pathway is believed to decrease ROS concentrations in order to keep a balance between ROS and the antioxidant potentials [22,23]. AST also acts against apoptosis by blocking p-ERK/ERK [12,13], cytochrome c, caspase3,9, and the Bax/Bcl2 ratio [4,24].
Nevertheless, considering the unsaturated structure of AST, it is highly susceptible to light, oxygen, and heat stress degradation. In addition, the poor water solubility and bioavailability of AST \([25–28]\) limit its efficacy in vivo \([29,30]\). So, investigating novel AST delivery system is necessary in order to solve these drawbacks.

To the best of our knowledge, this is the first review regarding the role of each signaling pathway in the pathogenesis of neurodegenerative disorders, including PD, AD, NP, depression, brain and spinal cord injuries, aging, and autism, and it is the first review regarding the auspicious effects of AST against neurodegeneration pathways as well as clarifying novel AST delivery systems, all together.

2. AST and Neurodegenerative Diseases

2.1. AST and Parkinson’s Disease (PD)

PD is an age-related disorder and the second most common cause of neurodegenerative disorders with a prevalence of 0.1–0.2% worldwide and 3% in people older than 80 years old \([31,32]\). PD is characterized by midbrain dopaminergic neurons lost, aggregation of \(\alpha\)-synuclein called Lewy bodies, and destruction of non-dopaminergic pathways leading to motor and non-motor dysfunction \([33,34]\).

Due to their low efficacy and undesirable adverse effects, conventional treatments of PD are quite challenging. Hence, discovering an effective and safe novel multi-target agent to combat PD is of great importance.

Neuroinflammation and oxidative stress have a major contribution in the pathogenesis of PD \([35,36]\). The inhibition of the mediators of these pathways plays an important role in preventing the disease progression, which is what AST does as a multi-target drug. In a rat model of homocysteine (Hcy)-induced hippocampal neurotoxicity and apoptosis, AST regulated ROS-mediated oxidative damage and mitochondrial dysfunction. AST also attenuated PI3K/AKT and mitogen-activated protein kinase (MAPK) pathways and thus, was used to tackle these neurological disorders, such as PD \([37]\).

AST acted through the SP1/NR1 and HO-1/NOX2 axis to inhibit MPP\(^+\) induced oxidative stress in PC12 cells \([38,39]\). MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) as a progressive cause of PD in experimental models \([40]\) affected the hydroxylase activity of tyrosine as an enzyme involved in dopamine biosynthesis \([41]\).

AST conserved substantia nigra from MPTP-induced dopaminergic neuronal loss in aged and young mice, but it was not able to protect against the loss of tyrosine hydroxylase induced by MPTP in aged mice. Therefore, Grimmig et al. considered aging as a critical factor in finding novel therapies for PD \([42]\).

In an in vitro study, Lee and colleagues reported that AST ameliorated MPP\(+\)-induced production of ROS in SH-SY5Y human neuroblastoma cells. This effect may be attributed to a decrease in \(\alpha\)-synuclein, caspase-3, and the Bax/Bcl-2 ratio and the increase of SOD, catalase, and tyrosine hydroxylase \([43]\).

Moreover, Liu et al. indicated that AST pretreatment inhibited 6-hydroxydopamine (6-OHDA) or DHA hydroperoxide (DHA-OOH)-induced apoptosis, intracellular ROS generation, and mitochondrial dysfunctions in dopaminergic SH-SY5Y cells through its antioxidant potential and mitochondria protection \([44]\). Also, AST inhibited 6-OHDA-induced apoptosis and mitochondrial dysfunction via blocking the phosphorylation of p38 MAPK and reducing caspase 3/9 and poly(ADP-ribose) polymerase \([45]\).

2.2. AST and Alzheimer’s Disease (AD), Cognition, and Memory

The brain has rich irrigation with blood vessels, very high oxygen consumption, and lower antioxidant capability, which it is very susceptible to oxidative damage \([46]\). AD is an age-related neurodegenerative disease, which is characterized by the overproduction and deposition of beta-amyloid peptide (A\(\beta\)) plaques and intracellular neurofibrillary tangles, and by a loss of neurons in the brain \([47,48]\). One of the main reasons for the development and progression of AD is the
oxidative stress [49,50]. AST is one of the few compounds that can cross the blood-brain barrier (BBB) in mammals, and going beyond this barrier could increase their antioxidant properties. The molecular mechanisms still are not elucidated but there are many types of research focused on neuronal apoptosis. Shen et al. reported that AST reduced ischemia-related injury in brain tissue, mainly through the inhibition of oxidative stress. It also protected neuroblastoma cells against Aβ-induced oxidative cell death through induction of the antioxidant enzyme HO-1 expression [51]. Later, Wen et al. investigated the neuroprotective effects of AST on glutamate-induced oxidative ex situ toxicity in a mouse hippocampal HT22 cells through Nrf2-dependent HO-1 expression [52]. The results indicated that AST is a promising biologically active compound for the treatment of neurodegenerative disorders such as AD. The amount of glutathione in the plasma has a correlation with the severity of cognitive dysfunction in AD patients [53]. Another study demonstrated that AST apparently showed a protective effect on L-glutamate-induced PC12 cell death mainly through the Bcl-2/Bax signaling pathway and, therefore, it could be considered a promising agent as prophylactic or remediation against neuronal disorders [54]. An experiment with double transgenic mice administrated with AST and its synthesized variant docosahexaenoic acid-acylated AST diesters (AST-DHA) for 2 months suggested that AST-DHA might be a potential therapeutic agent for AD. In the study, Radial 8-Arm Maze Test, Water Maze Test, Determination of Aβ Concentration, and western blot analysis was carried out [55].

Many studies aimed at exploring the relationship between diet and their effects on cognitive ability [56,57]. Hussein et al. reported the neuroprotective actions of AST and its high potential in human health and nutrition [58]. The contribution of fish oil in the process is an important and significant step in the protection of the nervous system and especially of the brain [59,60]. A protective function of AST in microcirculation and mitochondrial functions was demonstrated [15], which confirms its potential efficacy in several neurodegenerative diseases [61].

2.3. AST and Neuropathic Pain (NP)

NP is caused by a disease or a lesion in the somatosensory nervous system [62], with an estimated cost of 40 billion dollars per year in the U.S. [63]. Several destructive signaling pathways and mechanisms are involved in NP, mostly including neuromodulators (glutamate, and especially NR2B, gamma-aminobutyric acid (GABA), serotoninergic, and noradrenergic) and inflammatory agents (cytokines, prostaglandins, and reactive oxygen species) [64], which affect microglia and astrocytes activation, ion currents, and neuronal firing [65,66], as well as apoptosis [67].

Antidepressants and anticonvulsants are among the primary clinical alternatives for the management of NP [68]. Nevertheless, investigating novel multi-target pharmacological therapies for NP, which simultaneously target multiple destructive mediators with acceptable efficacy and safety, is of great importance.

As Sharma et al. reported, AST attenuated biochemical and behavioral alterations using in vivo models of NP. They found that AST decreased astrocytic activation. Thereby, glial fibrillary acidic protein (GFAP) afforded suppression and reduced oxido-nitrosative stress in vitro. Also, AST antagonized NR2B in silico and reduced thermal and mechanical allodynia in a rat model of chronic constriction injury (CCI)-induced NP [69]. In the same in vivo model of NP, AST prevented the increase in IL-6, IL-1β, and TNF-α in the spinal cord and hippocampus of mice [70]. Fakhri and colleagues confirmed the neuroprotective role of NR2B as a glutamate-gated channel, as well as the inhibitory effects of AST on NR2B and the glutamate-initiated signaling pathways in a rat model of compression spinal cord injury (SCI). It was also found that, besides improving neuronal damages, AST down-regulated TNF-α and p-p38MAPK, through which neuroinflammation and mechanical allodynia was inhibited [13]. We also confirmed the neuroprotective effects of AST in reducing the cold allodynia passed through the inhibition of p-ERK/ERK and the activation of p-AKT/AKT [12]. In a carrageenan-induced mice model of pain and paw edema, AST decreased thermal and mechanical allodynia, as well as the lipid peroxidation and myeloperoxidase enzyme in the paw [71].
Altogether, AST is introduced as an effective drug to combat NP. Additionally, since patients with chronic NP are at high risk of co-morbid depression [70], the need to investigate the antidepressive effects of AST is greatly raised.

2.4. AST and Depression

Depression is a common and complicated psychological condition for human health. As a major cause of mortality and morbidity, depression is predicted to be the main pathogeny of disability by 2030 [72]. Since the complex pathophysiology of depression is not yet completely known, the most suitable treatments are yet to be clarified [73]. In the treatment of depression, monoamine regulation still plays a crucial role and brings up tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), monoamine oxidase inhibitors (MAOIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and selective-norepinephrine reuptake inhibitors (SNERIs) [74,75], although most of them have low efficacy with potential adverse effects [76]. On the other hand, according to recent evidence, there is a close connection between depression and oxidative stress/inflammation, as non-monoaminergic pathways are involved in depression, which are now areas of active investigation. Besides, destructive intracellular pathways of oxidative stress and inflammation also underlie the etiology of depression and anxiety [77–79]. Altogether, investigating novel multi-target therapeutic agents for depression with acceptable safety and efficacy is still a medical need.

Several studies have reported the antidepressant-like effects of AST in different experimental models. As reported by Zhou and colleagues, AST prevented hyperglycemia-induced neuroinflammation contributing to depression. They also found that AST had an antidepressant-like effect by decreasing the level of IL-1β, IL-6, cyclooxygenase-2 (COX-2), cleaved caspase-3, and GFAP, and protecting neurons in the amygdala, hypothalamus, and hippocampus of mice [80]. In this context, Jiang et al. found that trans-AST ameliorated lipopolysaccharide (LPS)-induced depressive-like behaviors through the down-regulation of TNF-α, IL-6, and IL-1β and by antagonizing inducible nitric oxide synthase (iNOS), neuronal nitric oxide synthase (nNOS), and COX-2 expression in mice [81]. Chronic treatment with trans-AST also prevented co-morbid depression in mice, owing to their potent anti-inflammatory effects and involvement in the serotonergic pathway [70]. In this sense, the involvement of the serotonergic pathway in the pathogenesis of depression and related inhibitory effect of trans-AST were raised again [82].

In a rat model of depression, AST reverted the antagonistic and impairing effects of ethanol on cortical spreading depression in a dose-dependent [83], but not age-dependent [84], manner, which was attributed to the antioxidant effects of AST [83]. Moreover, Qiao et al. used a mice model of omethoate-induced depression and found that a combination therapy of AST and lithium chloride efficiently attenuated depressive-like behavior through the Akt/GSK3β/CREB signaling pathway [85].

As another crucial non-monoaminergic mechanism, the NMDA receptor and the glutamatergic pathway play destructive roles in the pathogenesis of depression. A type-specific NMDA receptor antagonist could offer more efficacy and fewer complications related to broader NMDA receptor blockers. Since AST blocks NR2B efficiently [13], it can be suggested as a strong anti-depressant drug.

2.5. AST and Central Nervous System (Brain/Spinal Cord) Injuries

Central nervous system (CNS) injuries, including brain and spinal cord injury, affect millions of individuals worldwide [86,87]. As complex processes of primary and secondary phases, CNS injury phases initiate temporary or permanent neuronal damages. Following the mechanical injury, the primary phase is characterized by direct death of cells followed by the secondary phase, consisting of inflammatory, oxidative, apoptotic, and other molecular pathways that cause further edema and damages to neuronal cells by inciting a breach in the BBB [88–90]. There are several destructive mediators that can be targeted by neuroprotective agents to prevent CNS injuries. However, there are still no sufficient data available regarding the improvement of post-CNS injuries.
In the context of brain injury, AST treatment attenuated early brain injury (EBI) after subarachnoidal hemorrhage (SAH) by reducing the brain edema, BBB disruption, and caspase3. This neuroprotective effect of AST has been attributed to its strong antioxidant property by decreasing malondialdehyde (MDA) and increasing glutathione (GSH) and SOD in rodent models [90]. In an in vivo study, AST significantly prevented \( \text{H}_2\text{O}_2 \)-induced apoptosis, improved neurological deficit, and diminished the infarct volume, as well [91]. Also, AST suppressed oxygen-glucose deprivation (OGD)-induced oxidative stress by upregulating the protein expression of HO-1, Hsp32, and Hsp90 in SH-SY5Y cells. Thus, it is confirmed that the neuroprotective effects of AST in CNS damages are also related to its antioxidant effects [92].

Following EBI in SAH model, AST positively attenuated the cortical expression of NAD (P) H: quinone oxidoreductase 1 (NQO-1), HO-1, and glutathione S-transferase-α1 (GST-α1) through the antioxidant pathway named Nrf2-ARE at both mRNA and protein levels. Additionally, AST ameliorated BBB disruption, brain edema, apoptosis, and neurological dysfunction in this context [93]. Considering that apoptosis plays a crucial role in the pathogenesis of EBI, AST considerably increased the phosphorylation of Akt and Bad levels, which led to a reduction in apoptosis and caspase-3 levels following SAH [94]. AST ameliorated mitochondrial membrane potential, cerebral vasospasm, and mitochondria-associated neuronal apoptosis by reducing caspase-3, the Bax/Bcl-2 ratio, and cytochrome c in the prefrontal cortex post-SAH [95].

Zhang et al. found that treatment with AST prevented SAH injury by inhibiting the toll-like receptor 4 signaling pathway and increasing sirtuin 1 and the subsequent inflammatory response, both in vivo and in vitro [96]. This indicated the role of inflammation, besides oxidative stress and apoptosis, in CNS injuries. In a rat model of SAH, AST down-regulated matrix metallopeptidases-9 (MMP-9) which was attributed to a decrement in the level of infiltrating neutrophils, activated microglia, TNF-α, and IL-1β [97].

Recent advancements have also clarified the contribution of \( \text{Na}^+/\text{K}^+/2\text{Cl}^- \) co-transporters (NKCCs) and aquaporins (AQPs) to brain edema during traumatic brain injury (TBI). Following TBI, AST attenuated AQP4/NKCC1-level in mice brain tissue [98]. According to Zhang and colleagues, AST down-regulated NKCC1 expression through the nuclear factor-kB (NF-κB) pathway, which mediates pro-inflammatory factors, and also protecting astrocytes against TBI [99].

In a rat model of SCI, Fakhri et al. reported that AST down-regulated NR2B, TNF-α, and p-p38MAPK, and it also preserved the tissue and neuronal damages and improved the sensory-motor function following a rat model of compression SCI [13]. We also found that AST increased and decreased the protein expression ratio of p-AKT/AKT and p-ERK/ERK, respectively, following SCI [12].

### 2.6. AST and Aging

The oxidative stress increases with aging and the brain become significantly more vulnerable to neurodegenerative disease [100,101]. The deficits in memory formation in older individuals caused by oxidative stress affect synaptic plasticity in neural networks in the hippocampus by protecting D-serine-dependent NMDA receptor activation. Hippocampal neurogeneration is associated with learning and memory processes [102]. Another study in mice showed that hippocampal oxidative stress is age dependent [103]. The reaction of young and aged animals to AST treatment on brain oxidative markers showed that there are no significant differences among them, and AST improves all types of oxidative markers in the six studied brain regions—namely the frontal cortex, striatum, parietal cortex, hypothalamus, hippocampus, and cerebellum [104]. The effects of AST on the aging female and male rat brains have been analyzed and the results obtained showed the gender-related differences [105].

Researchers studied the inhibition of oxidative injury of biological membranes by AST and found that its efficacy is higher than vitamin E, which is associated with the mitochondrial theory of aging [106]. This property highlights its unique potential to combat aging [4,107]. Hussein et al.
marked the properties of AST for the prevention of age-related macular degeneration [58], and Wu et al. reported its effect on inhibition of apoptosis and alleviation of injury in the brains of aging rats [108].

Topically applied, AST affected aging skin with a visible wrinkle reduction. There are some clinical studies supporting this statement [109]. Yamashita found that the combined use of a dietary supplement containing AST and tocotrienol from palm oil resulted in a significant reduction of fine wrinkles [110]. On the other hand, a single-blind placebo-controlled study showed that dietary supplement containing only AST with a dose of 4 mg per day led to significant improvements in human skin characterized by elasticity during a dermatologist’s visual assessment [109]. The combination of oral supplementation with topical application resulted in significant improvements in skin wrinkles, age spot size, elasticity, skin texture, moisture, the content of the corneocyte layer, and the corneocyte condition [111].

2.7. AST and Autism

As a neurodegenerative disease with an increasing prevalence, autism is characterized by impairments in communication, social interaction, behavior, and regular activities [112–114]. Age-related and progressive neuronal pathology and neuronal loss in cerebellar Purkinje [115] or amygdala [116] cells in individuals with autism [117] are all representative of a neurodegenerative process [118–121]. There is also a close link between autism and the release of proinflammatory cytokines [122,123]. Thus, AST is a multi-target anti-neuroinflammatory drug that can be used against autism.

Besides, given the importance of oxidative stress in the pathogenesis of autism [124–127], AST as the strongest antioxidant among the carotenoid pigments [9] can be used to combat autism, as Ornoy et al. also reported [128]. On the other hand, the plasma concentrations of exogenous antioxidants, such as carotenoids, are insufficient in autistic adolescents and children, which confirms the role of oxidative stress in the pathogenesis of autism and the inhibitory effects of carotenoids [129].

AST increased the paw withdrawal latency, evaluated by a hot plate test, and improved the behavioral disorders, assessed by the social interaction and open field tests, in a mice model of pre-natally valproic acid-induced autism. In the same context, AST treatment also reduced oxidative stress through the reduction of nitric oxide and lipid peroxidation and the increment of catalase activity [104]. Frutos et al. reported that due to their anti-oxidative and anti-inflammatory properties, carotenoids are introduced as potential foods against autism [130].

Considering the role of oxidative stress and neuroinflammation signaling mediators in the pathogenesis of autism, each of these mediators can be a target to combat autism. Even though there is not enough evidence regarding the effects of AST on autism, it can be suggested as an auspicious neuroprotective agent against autism due to its inhibitory role on neuroinflammation and oxidative stress.

Figure 2 shows the neuroprotective mechanisms of AST for combating neurodegenerative diseases (Figure 2).
As a division of nanotechnology, nanomedicine uses biocompatible and biodegradable nanoaggregates and submicron-sized nanoparticles to target pharmacokinetics, administration routes, and bioavailability of drugs in medicine [131].

AST is a lipid-soluble carotenoid with low bioavailability that is partly absorbed by intestinal cells. It displays poor dispersibility and water solubility in aqueous solutions and is susceptible to light, oxygen, and heat stress degradation [25–28]. In spite of the strong in vitro neuroprotective effects of AST, the lack of an appropriate AST-delivery system to pass through BBB fails to show the same responses in vivo. Such characteristics of AST have prevented it from being widely used in biomedical or pharmaceutical applications. On the other hand, various approaches have so far been applied to formulate AST into a novel drug delivery system in order to increase its stability, solubility, and bioavailability, and to prolong its shelf life [29,30]. In addition to low bioavailability and lack of an appropriate drug delivery system, high thermostability, instability, and lipophilicity of AST have caused its antioxidant efficacy to be failed in clinical trials. Thus, developing an appropriate drug delivery system capable of overcoming these limitations is of great necessity. However, there are promising ways to overcome these limitations and to improve the performance of pharmaceuticals to prepare a suitable nanoformulation of AST. In this regard, different formulations of AST-loaded lipid-based carriers (LBCs), polymeric systems, and inclusion complexes have been provided to potentiate its effect [132].
As an auspicious delivery system, LBCs offer to enhance the stability and bioavailability of active pharmaceutical ingredients (API) while allowing a controlled release [132,133] achieved by a surface charge or adsorption of a layer of polymer or surfactant. LBCs consist of oil in water (O/W) nano/microemulsions, oil-loaded solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and micelles [134].

As dispersions of small spheroid within an aqueous medium, O/W microemulsions are thermodynamically stable, while nanoemulsions are unstable colloids [134,135]. The bioavailability of AST nanoemulsions has been reported to increase through the elevation of its oxidative and physical stability [136,137]. SLNs are mixtures of O/W nano/microemulsions with the high-ordered inner crystalline structure of lipid phase. SLN-AST is shown to possess unique properties such as large surface area, high drug loading, small size, and a wide spectrum of biodistribution, which, in return, realizes the goals of site-specific and controlled drug delivery as a promising strategy for efficient delivery of hydrophobic drugs. It offers more potential neuronal applications for passing through BBB to protect the brain from oxidative stress and to provide valuable support for brain health [138,139].

As reported by Bhatt et al., SLN-AST was shown to be an effective treatment to tackle oxidative stress-induced neurodegeneration in pheochromocytoma-12 cell line [139]. AST nanoemulsion also plays a momentous role in the stimulation of oxidative stress and mitochondrial-mediated apoptosis in cancer cells [140]. So, mitochondrial-targeted therapy via novel delivery systems could potentiate the effect of AST. Since drug-loaded nanoemulsion is a lipophilic molecule, it is likely to be localized to the membrane, followed by the mitochondria and nucleus [141]. Such drug delivery systems are implemented to selectively target mitochondria, and the targeted therapy was confirmed by apoptotic changes, mitochondrial membrane potential, and intracellular changes of membrane ROS [140].

To compensate for the low bioavailability and capacity of drug loading and release in SLN, NLCs were developed with a less-ordered inner crystalline structure allowing higher bioavailability and loadings of the lipophilic API with easier scale-up [142–145]. Indeed, NLCs, as more superior options than other colloidal carriers, present a remarkable capability to preserve, stabilize, and amplify the antioxidant capacity of AST [132,133,146,147]. Rodríguez-Ruiz et al. reported that ASTMCO2-NLCs could be potentially introduced as excellent candidates for developing new platforms applications for medical devices, as well as antioxidant delivery systems for nutraceuticals [146]. Tamjidi et al. showed no drastic effects of ionic strength, heat, pH, and simulated gastric juice on the chemical stability of AST-loaded NLCs [148]. Several other studies also suggested NLCs as a successful AST-delivery system [29,149]. Although NLCs improve the functionality of AST from different aspects, the key role of emulsifiers in the system composition cannot be neglected [150,151], indicating the strong effect of the emulsifier on the bioavailability of AST-loaded NLCs [152].

Along with SLNs and NLCs, nanoliposomes as nano-scaled colloid systems made from the dispersion of amphiphilic lipids in aqueous solvents are considered to be other successful lipid base carriers in delivery systems [153]. Nanoliposomes benefit from such characteristics as higher membrane penetration and bioavailability, as well as superior potentiality to be used for specific drug targeting systems [154]. Pan et al. revealed that AST-loaded nanoliposomes increased the membrane micropolarity and decreased the membrane fluidity in order to attenuate the membrane structural properties [153]. AST-loaded liposomes functioned as a promising drug delivery system to treat hepatotoxicity [155] with increased bioavailability [155,156]. According to Peng et al., liposome encapsulation also elevated the stability, transportability, and antioxidation properties of AST [156]. The potential of liposomal encapsulation to enhance the radical scavenging effects of AST has been shown by other studies as well [157–159].

Polymeric systems have also been recently used as other novel delivery systems to enhance the solubility, protection of the biological activity, physicochemical stability, and the antioxidant effects of AST, and also to control its release. Due to their biodegradability and biocompatibility properties, chitosan [160,161] and alginate [134,162–164], as natural polysaccharides, are used in polymeric delivery systems of AST microencapsulation, leading to higher cell uptake and antioxidant effects of AST.
The absorption profile of polymeric nanosystems of AST is affected by its shape, particle size, and surface properties, which could be used to handle the release of AST [164].

Another upcoming method for AST delivery is to use the inclusion complex formation of cyclodextrin. It is a natural macrocyclic oligosaccharide with a hydrophilic outer surface and a lipophilic cavity used to enclose lipophilic molecules like AST [165]. The involved non-covalent guest–host associations result in the controlled release of the guest, increase of its hydrophilicity, stability against light, heat, and oxygen [166], and bioavailability [167], as well as an improvement in the antioxidation potential of the guest [168]. In several studies, cyclodextrin has shown the potential to increase the stability, hydrophilicity, bioaccessibility [169,170], and antioxidant activity of AST [168]. It is worth mentioning that cyclodextrin inclusion formation gives more hydrophilicity activity to AST, compared to NLCs and polymeric delivery systems. Figure 3 indicates novel AST delivery systems as well as their effects on AST properties (Figure 3).

![Figure 3. Novel AST delivery systems. O/W: oil in water, W/O: water in oil, SLNs: solid lipid nanoparticles, NLCs: nanostructured lipid carriers, and AST: astaxanthin.](image)

From the diagnostic point of view, AST nanoparticles could also be used for diagnostic purposes. Bharathiraja et al. reported the potential of AST gold nanoparticles in the photo-based diagnosis of cancer in the near infra-red range [171].

In general, the novel delivery systems of AST, including lipid carriers, mitochondrial targeted, polymeric, and cyclodextrin inclusion systems, could be considered as new ways to potentiate the effects of AST by improving its stability, hydrophilicity, and antioxidant capacity to be used in several diseases (i.e., neurodegenerative disorders).

4. Conclusions and Future Perspective

AST is an oxy-carotenoid with potential effects on healthcare. Although most of the studies have considered the protective effects of AST on various diseases, recent studies have focused more on their neuroprotective effects. As a multi-target neuroprotective agent, AST affects multiple mechanisms of
action to tackle complex pathophysiological mechanisms of neurodegenerative diseases, mainly based on its anti-inflammatory, antioxidant, and antiapoptotic effects. All neuroprotective pharmacological mechanisms of action of AST are highlighted in the current review. On the other hand, the lack of an appropriate drug delivery system of AST has caused its efficacy to be failed in clinical trials. So, investigating an appropriate AST delivery system in order to solve this drawback is of great importance.

Such reports will provide novel applications of AST in the prevention, management, and treatment of neurodegenerative diseases, as well as investigating the most potential novel AST delivery system in clinical trials. Additional studies are needed to elucidate the precise pathophysiological pathways involved in neurodegeneration, and to clarify the potential neuroprotective effects of appropriate AST formulations on humans.

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Astaxanthin extends lifespan via altered biogenesis of the mitochondrial respiratory chain complex III

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Running title: “Astaxanthin affects mitochondria and life-span”
Abstract

Astaxanthin is a keto-carotenoid produced in some bacteria and algae, which has very important industrial applications (i.e., in cosmetics, coloring additive in aquaculture and as a dietary supplement for human). Here, we analyzed the molecular basis of Astaxanthin-mediated prolongevity in the model organism, Caenorhabditis elegans. The increased lifespan effects of Astaxanthin are restricted in C. elegans to the adult phase and are uninfluenced by various other carotenoids tested. Genetic analyses indicated that the Astaxanthin-mediated life-extension relies on mitochondria activity, via the Rieske iron-sulfur polypeptide-1 (ISP-1), but is not influenced by the functions of other known longevity-related gene-loci, including CLK-1, DAF-2, DAT-16, EAT-2, GAS-1 GLP-1 or MEV-1. Biochemical analyses of native respiratory complexes showed that Astaxanthin affects the biogenesis of holo-complex III (and likely supercomplex I+III, as well). Effects on holo-CIII assembly and activity were also indicated by in-vitro assays, with mitochondria isolated from worms, rodents, human and plants, which were treated with Astaxanthin. These data indicated a cross-species effect on the oxidative phosphorylation (OXPHOS) machinery by the carotenoid, and provide with further insights into the molecular mechanism of animals longevity extension by Astaxanthin.

Keywords: Caenorhabditis elegans, Carotenoids, Astaxanthin, median lifespan; mitochondria, OXPHOS, Complex III, Rieske iron-sulfur protein,

Significance Statement

Astaxanthin is a widely consumed pigment by animals and human. In this study we find that Astaxanthin, but not other tested carotenoids, significantly extends the lifespan of animals by affecting respiratory complex III (CIII) biogenesis of the mitochondria, in plants, C. elegans, rodents and human. We further propose a model to try explaining this effect of astaxanthin on animals’ longevity.
Introduction

The nematode *Caenorhabditis elegans* is a leading model organism for studying the biology of aging, and for identifying new pharmacological targets for extended longevity and the treatment of aging-related diseases in animals (Chen et al. 2014). Genetic studies indicated three major aging-related mechanisms in *C. elegans*. These include the insulin pathway, the dietary restriction system, and the mitochondrial respiration system, which were all shown to affect the lifespan of worms and other animals (Amrit et al. 2014, Collins et al. 2008). Noteworthy, the majority (>80%) of the *C. elegans* proteome was shown to share homology with known human genes (Henricson et al. 2004, Lai et al. 2000), thus suggesting common aging-related mechanisms between *C. elegans* and human.

Among the key factors that affect longevity in worms is oxidative damage, generated by reactive oxygen species (ROS). Induced ROS level was shown to induce early aging in various animals and plants (Paital et al. 2016, Singh et al. 2016). Consequently, strong antioxidants as resveratrol, coenzyme Q10 (ubiquinol or 2,3-dimethoxy-5-methyl-6-decaprenyl benzoquinone) and carotenoids as Astaxanthin can extend the lifespan of *C. elegans*, presumably by scavenging ROS and lowering the levels of oxidative stress (Chen, et al. 2014). Carotenoids comprise a large class of natural antioxidant pigments that are synthetized and accumulate in photosynthetic organisms, various bacteria and some fungi (Cazzonelli 2011, Rodriguez-Concepcion et al. 2018). These isoprenoid compounds are also essential components of the photosynthetic machinery, and are associated with anti-oxidative reactions in bacteria, fungi, algae and plants. In animals, carotenoids that are consumed from the food play important roles in the antioxidant defense mechanism (Fiedor and Burda 2014).

Astaxanthin (i.e., 3,3-dihydroxy-ß,ß-carotene-4,4-dione) is an orange-red pigment containing 13 conjugated double bonds (Supplementary Fig. S1). As other carotenoids, Astaxanthin is a lipid-soluble pigment, which has self-limited absorption orally, with no known toxic syndromes (Barros et al. 2014). Astaxanthin is naturally produced by some marine bacteria, algae and plants. Many red-colored marine animals (e.g., salmons, shrimps) and a few bird species (e.g., flamingo) obtain the Astaxanthin orally, through the food chain (Ambati et al. 2014). As a strong antioxidant, Astaxanthin was utilized as a common antioxidant agent and as a dietary supplement, intended for human, animal and aquaculture consumption. Astaxanthin has been implicated in numerous health benefits in humans, including reduction in cardiovascular diseases, enhancement of the immune response and reduction in the occurrence of various cancers. (Ames 2018, Zhang and Wang 2015, Zhang et al. 2016). Astaxanthin is
obtained industrially by either chemical synthesis or by extraction from algae or bacteria, both of which are approved by the U.S. Food and Drug Administration as food, feed or coloring additives (Raposo et al. 2015, Zhang and Wang 2015). Plant-derived Astaxanthin is generally recognized as safe by the FDA, meaning it can be sold as a dietary supplement (Zhang and Wang 2015), although in the United States is restricted to be used only as a colorant agent to animal feed. Currently, there is great interest around Astaxanthin due to its biochemical characteristics, mainly as a potent antioxidant, which is approximately 10 times more effective than β-carotene or lutein and about 100 times than α-tocopherol (Higuera-Ciapara et al. 2006, Rao et al. 2015). These studies further suggest that Astaxanthin affects cellular metabolism and protects against fatty acid oxidation.

Astaxanthin was previously shown to extent the lifespan of both flies and worms. *Drosophila melanogaster* mutants in CuZn-superoxide dismutase (SOD1) and Mn-superoxide dismutase (SOD2), as well as mutant-lines with reduced levels of SOD1, SOD2 and catalase, show significant lifespan extension and amelioration of age-related decline in motility when are fed with *Haematococcus pluvialis* (a microalga which accumulates high-levels of Astaxanthin) (Huangfu et al. 2013). Likewise, feeding *C. elegans* with Astaxanthin also extends the mean lifespans of both wild-type and long-lived mutant age-1 animals (Kashima et al. 2012, Yazaki et al. 2011). The molecular basis of astaxanthin-mediated prolongevity effects seen in both *D. melanogaster* and *C. elegans* remained unknown, but it was anticipated to be mediated through the scavenging of reactive oxygen species (ROS) produced in these animals by the carotenoid compound.

In this study we show that the lifespan of *C. elegans* fed with Astaxanthin increases by about 20%, which is in accordance with previously publish data showing the effects of Astaxanthin on the mean lifespans of the warm (Liu et al. 2016). Our data further indicate that the effects of Astaxanthin are independent of the insulin/IGF or dietary restriction of germline pathways, and provide with evidence that the extended longevity effects on *C. elegans* is mediated by the disassembly or altered biogenesis of the native respiratory complexes III2 and presumably the supercomplex I+III2. The effects of Astaxanthin on the mitochondrial OXPHOS system seems universal, as was also apparent by ‘in-organello’ assays with mitochondria preparations from plant, *C. elegans*, mice rat and human. These results are of great importance as they offer with novel insights into the molecular basis of longevity extension by Astaxanthin, and may also lead to developing new generation of drugs based on carotenoids to prolong lifespan in animals and human.
Materials and Methods

*C. elegans* strains

*C. elegans* strains maintenance was performed under standard conditions, essentially as described (Brenner 1974). N2 (Wild-type), *daf-2* (e1370), *daf-16* (mu178), *glp-1* (or178), *eat-2* (ad1116), *clk-1* (e2519), *mev-1* (kn1), *gas-1* (fc21) and *isp-1* (qm150) strains were used in this study. All strains were obtained from the *C. elegans* Genome Center (CGC) and were out-crossed with wild-type (N2) animals.

Lifespan assays

Lifespan assays were performed essentially as described previously (Bar *et al.* 2016). Synchronized *C. elegans* animals were grown on NGM (K$_2$HPO$_4$, KH$_2$PO$_4$, NaCl, Bacto peptone, Agar, Agarose, MgSO$_4$, CaCl$_2$, and Cholesterol) plates containing one of the following bacterial strains: Wild type *Paracoccus marcusii*, which are accumulating high-levels of Astaxanthin, *P. marcusii* ΔcrtB mutant which lacks carotenoids, *Escherichia coli* OP50 strain (Taxonomy ID 637912), recombinant *E. coli* cells carrying the pASTA plasmid for the expression of Astaxanthin (Cunningham and Gantt 2007). The carotenoid composition in different bacterial strains used in this work is indicated in Supplementary Table S1. In addition, the worms were also grown in the presence of *E. coli* cells in agar-plates supplemented with Astaxanthin (extracted from the algae *Haematococcus pluvialis*) solubilized in 5% DMSO (w/v), and agar-plates containing 5% DMSO with *E. coli* (OP50) cells, as control. Experiments were performed at 20°C, with the exception of *glp-1* and *daf-2* mutant-lines which were conducted at 25°C. In the *daf-2* experiments, the worms were grown at 16°C until the L4 stage, and then transferred to 25°C. For each experiment, 80 adult animals were placed on four separate NGM-plates. Animals were considered dead when they no longer responded to gentle prodding with a platinum wire. Scoring was performed every day. For all lifespan experiments, assays were repeated at least twice.

Measurements of carotenoids concentration

Wild-type *P. marcusii* or *E. coli* producing Astaxanthin were plated on NGM media, harvested from the plates and resuspended in 2 ml of DDW. The cells were then washed once in 2 ml DDW. The bacterial suspension (1.8 ml) was incubated at 105°C for 24 h, after which the weight of the bacteria was measured. For carotenoid extraction, after washing in DDW, the cells were resuspended in 1 ml of acetone and incubated at 65°C for 10 min in the dark. The
samples were centrifuged again at 13,000 xg for 10 min and the acetone supernatant, containing the pigments, was placed in a clean tube. The pigment extract was dried with N₂ and then stored in the freezer at -20°C. Acetone-dissolved pigment extracts were analyzed by high performance liquid chromatography (HPLC), essentially as previously described (Neuman et al. 2014, Ronen et al. 1999), using a Waters 996 photodiode array detector. Carotenoids were identified based on their characteristic absorption spectra and typical retention times, which corresponded to standard compounds (Isaacson et al. 2002). For Astaxanthin extraction, wild-type algae *Haematococcus pluvialis* (kindly provided by Prof. Sammy Boussiba, BGU) were grinded with a mortar and pestle in 5 ml of acetone. The cells were incubated at 65°C for 10 min in the dark, and centrifuged at 13,000 xg for 10 min. The supernatant, containing acetone-soluble pigments, was placed in a clean tube, dried with a stream of N₂ and then subjected to HPLC analysis.

**Crude mitochondrial preparations from *C. elegans* cells**

Mitochondria were isolated from *C. elegans* according to the method described in Grad et al. (2007). Bleached embryos were grown at 20°C, on NGM plates seeded with either *P. marcusii* expressing Astaxanthin or *E. coli* (OP50). Before extraction, worms of the specified stages were washed off plates with sterile M9 solution (42 mM Na₂HPO₄, 22 mM KH₂PO₄, 86 mM NaCl, and 1 mM MgSO₄·7H₂O). The worms were pelleted by centrifuging at 3,000g for 5 min, and then resuspended in 10 ml of ice-cold isolation buffer (IB) (210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 5 mM Tris-HCl pH 7.4) in the presence of protease inhibitors. The worms were homogenized using a glass homogenizer for 15 strokes. The homogenate was collected in a 50 ml tube and 10 ml of IB was added. Then tubes were centrifuged at 750 g for 10 min at 4°C. The clear supernatant was transferred to a new tube, and centrifuged at 12,000 g for 10 min at 4°C to pellet the mitochondria. The organellar fraction was washed once in 10 ml ice-cold IB (12,000g for 10 min at 4°C), and the mitochondrial pellet was aliquoted and stored at -80°C.

**Mitochondrial extraction from cauliflower**

Mitochondria were isolated from cauliflower (*Brassica oleracea* var. botrytis) inflorescences, which allows the purification of large quantities of highly enriched organellar preparations (Keren et al. 2009, Neuwirt et al. 2005, Sultan et al. 2016). About 1.0 kg fresh weight inflorescences were cut off from the stems and kept overnight (about 16 h) in cold water (4°C). In the following morning, the inflorescences were ground with ice-cold extraction buffer (0.9 M mannitol, 90 mM Na-pyrophosphate, 6 mM EDTA, 2.4% PVP25 (w/v), 0.9% BSA (w/v), 9 mM cysteine, 15 mM glycine, and 6 mM β-mercaptoethanol; pH 7.5). Mitochondria were
recovered from the extract by differential centrifugations and purified on Percoll gradients. Following fractionation, the mitochondria were pelleted, resuspended in a small volume of wash buffer (0.3 M mannitol, 10 mM K-phosphate, 1 mM EDTA, pH 7.5), aliquoted and stored frozen at -80°C. For the isolation of mitochondria from Arabidopsis, we used 2-week-old seedlings grown on MS-plates. The enriched organelle preparation was performed essentially as described in (Keren et al. 2012). For analysis of Astaxanthin effects, the mitochondria were incubated with Astaxanthin dissolved in DMSO or DMSO alone for 5 min on ice.

**Mitochondrial enriched preparation from mammals**

Mitochondrial enriched fraction, was prepared from human (fibroblast) and mouse (heart) by teflon-glass homogenization and differential centrifugation in sucrose buffer (250 mM Sucrose, 10 mM Tris pH 7.4, 50 µg/ml heparin) as described (Saada et al. 2003). For enzymatic activities, mitochondria were pre-incubated with Astaxanthin or DMSO for 5 min on ice prior to assay.

**Blue native electrophoresis for isolation of native organellar complexes**

Blue native (BN)-PAGE of mitochondrial fractions was performed according to the method described by (Eubel et al. 2003). Mitochondrial pellets were solubilized with n-dodecyl-ß-maltoside (1.5% [w/v]) in ACA buffer (750 mM amino-caproic acid, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.0), and then incubated on ice for 30 min. The samples were centrifuged 8 min at 20,000g, and Serva Blue G (0.2% [v/v]) was added to the supernatant. The samples were then loaded onto a native 4% to 16% gradient gel. For non-denaturing-PAGE-Western blotting, the gel was transferred to a PVDF membrane (Bio-Rad) in Cathode buffer (50 mM Tricine and 15 mM Bis-Tris-HCl, pH 7.0) for 16 h at 4°C (const. 40 mA). The membrane was incubated with various antibodies, as indicated for each blot, and detection was carried out by chemiluminescence assay after incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody.

**SDS-PAGE gel electrophoresis**

Organellar protein concentration was determined by the Bradford method (BioRad, Catalog no. 5000201) according to the manufacturer's protocol, with bovine serum albumin (BSA) used as a calibrator. For immunoassays, an aliquot equivalent to 600 µg *C. elegans* mitochondrial proteins was suspended in sample loading buffer (Laemmli 1970) and subjected to SDS-PAGE (at a constant 100 V). Following electrophoresis, the proteins were transferred to a PVDF
membrane (BioRad, Catalog no. 1620177) and incubated overnight at 4°C with various primary antibodies (Table S2). Detection was carried out by chemiluminescence assay after incubation with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody.

Assessment of mitochondrial respiratory chain enzymatic activities
In-gel activity stains of mitochondrial respiratory complexes I, II and IV was carried out according to (Eubel et al. 2005). Following BN-electrophoresis, the gels were washed several times with DDW. For Complex I activity staining, the BN-gels were incubated in CI-activity assay solution (100 mM Tris-HCl, pH 7.4, 0.14 mM NADH, 1 mg/ml NBT solution), for 10 to 30 min until the purple staining of CI bands became visible in the gel. Complex II activity staining was carried out with 50 mM KH₂PO₄ buffer (pH 7.4), 84 mM succinate, 0.2 mM PMS and 2 mg/ml NBT solution. For complex IV activity staining, the BN-gels were incubated in 10 mM KH₂PO₄ buffer (pH 7.4), 1 mg/ml DAB solution and 0.2 mg cytochrome c. Reactions were stopped in fixing solution containing 15% (v/v) ethanol and 10% (v/v) acetic acid. All steps were carried out at room temperature. Respiratory chain activities and citrate synthase activity assayed by spectrophotometric methods at 37°C(Kirby et al. 2007, Shufaro et al. 2012). Briefly, complex I was measured as rotenone sensitive NADH-CoQ reductase at 340 nm in the presence of coenzyme Q₁. Complex II was measured as succinate dehydrogenase (SDH) based on the succinate-mediated phenazine methosulfate reduction of DCPIP (dichloroindophenol indophenol) at 600 nm. Complex II + III was measured as thenoyltrifluoroacetone sensitive succinate cytochrome c reductase at 550 nm. Complex III was measured as antimycin sensitive ubiquinol cytochrome c reductase at 550 nm Complex IV. (cytochrome c oxidase) was measured by following the oxidation of reduced cytochrome c at 550 nm. Citrate synthase (CS), an ubiquitous Krebs cycle enzyme, serving as a control, was measured in the presence of acetyl-CoA and oxaloacetate by monitoring the liberation of CoASH coupled to 5',5-dithiobis (2-nitrobenzoic) acid (i.e., Absorbance at 412 nm).

Respiration activity
Oxygen consumption (O₂-uptake) measurements were performed with a Clarke-type oxygen electrode (Oxytherm System, Hansatech Instruments, Norfolk, UK), and the data feed was collected by Oxygraph-Plus software, essentially as described previously (see e.g., (Shevtsov et al. 2018). The electrode was calibrated with O₂-saturated water, and by depletion of the oxygen in the electrode chamber with the addition of excess sodium dithionite. Total respiration was measured at 25°C in the dark, following the addition of 250 µL C.elegans suspension from
different treatments (± Astaxanthin) and mutant-lines to a 2.25 ml of M9 solution in the respiration chamber. The *C. elegans* suspension was prepared by 1/16 dilution of dense packed worms from 5 plates in M9 buffer. Oxygen consumption was also measured in the presence of various inhibitors, including Rotenone (Rot), Antimycin A (Anti A) and potassium cyanide (KCN).

**Statistical Analyses**

Lifespan curves were analyzed by plotting Kaplan-Meier survival curves (Goel *et al.* 2010), and by conducting Log-rank tests (Mantel 1966). Mean lifespan data was compared using Log-rank test with multiple comparisons test, OASIS-2 (Han *et al.* 2016). Differences with P-value smaller than 0.05 were considered as statistically significant.

**Results**

**Feeding adult *C. elegans* with Astaxanthin cause significant extension in their lifespan**

Synchronized *C. elegans* animals were grown for 5 days at 20°C on NGM plates containing *P. marcusii* cells (expressing Astaxanthin) or in the presence of *P. marcusii* ΔcrtB mutant-line (lacks carotenoids expression). Following 5 days, the intestine of the worms was cleaned by transferring the animals to new NGM plates, containing *E. coli* (OP50) cells, for additional 24 hours (Images of *C. elegans* before and after intestine cleaning procedure are provided in Supplementary Figure S2). Under these conditions, the average lifespan of wild-type *C. elegans* (N2), grown on agar plates and fed with *Paracoccus marcusii* (containing on average 716 g Astaxanthin per gram of dry material), was about 20% longer (i.e., 24 Days vs. 20 Days) than *C. elegans* fed with *P. marcusii* ΔcrtB mutant that lacks carotenoids (P-value = 0.0038) and about 45% longer (24 Days vs. 17 Days) compared with those of *C. elegans* fed with *E. coli* OP50 cells (P-value = 0.00003) (Fig. 1). Likewise, feeding *C. elegans* with a recombinant *E. coli* cell-line which produces Astaxanthin (pASTA, 146 g Astaxanthin per gram of dry material), was also sufficient to significantly increase the lifespan of the worms by about 20% (P-value = 0.0114, Fig. 1B). Feeding *C. elegans* with Astaxanthin extracted from the algae *Haemayococcus pluvialis* and placed on *E. coli* OP50 strain increased the average lifespan by ~20% (P-value = 0.0173, Fig. 1C). No effect on the lifespan was observed in *C. elegans* fed with *P. marcusii* strains expressing the carotenoids Zeaxanthin (P-value = 0; *P. marcusii* ΔcrtW, Fig. S2 and Table S1), while Lycopene (*P. marcusii* ΔcrtY, Table S1) was found to be toxic to the animals (animals arrested at the L2 stage).
Next, we determined the developmental period where Astaxanthin has the most notable effect on the lifespan of *C. elegans* (see Fig. S3). For this purpose, we used four different feeding conditions. These include animals that were fed from the larval L1 until adulthood with Astaxanthin (i.e., in the presence *P. marcusii*), and later transformed to new plates lacking Astaxanthin (i.e., plates containing *P. marcusii ΔcrtB*) (Fig. S3, L1-Mature +Asta, Adult -Asta). The second condition involved animals from the larva L1 stage until adulthood that were fed with *P. marcusii ΔcrtB* (lacks Astaxanthin) and later transferred to new plates contacting wild-type *P. marcusii* (produces Astaxanthin) (Fig. S3, L1-Mature -Asta, Adult +Asta). In addition, as controls we also used worms that were fed during their entire lifespan on either *P. marcusii* wild-type (Fig. S3, +Asta) or *P. marcusii ΔcrtB* (Fig. S3, -Asta).

As indicated Figure S3, feeding animals that are only at their adult stage (Fig. S3, L1-Mature -Asta, Adult +Asta) with Astaxanthin resulted with an increase in lifespan which was similar to that of animals fed with Astaxanthin throughout their entire life (P-values <0.05). However, no significant change in longevity was apparent when the animals were fed with Astaxanthin from the larval L1 stage until young adulthood, but were avoided from being exposed to the pigment, during their adult stage (Fig. S3, L1-Mature +Asta, Adult -Asta). Similarly, no increase in lifespan was seen when the animals are grown on *P. marcusii ΔcrtB* cells lacking any carotenoids.

We also noted that *C. elegans* animals fed with *P. marcusii* showed a strong red-orange color of the gut (Fig. S4, left panel). Feeding *C. elegans* with *P. marcusii* cells expressing Astaxanthin followed by 24 hours feeding with *E. coli* (OP50) resulted with a strong decolorization of the guts, but some residual red color was still observed in the bod cells (Fig. S4, middle panel). We therefore hypothesize that feeding *C. elegans* at the adult stage with Astaxanthin resulted with the accumulation of Astaxanthin in the cells, an effect that likely correlates with the observed extension in the lifespan.

The effects of Astaxanthin on *C. elegans* longevity are tightly associated to the mitochondria-aging pathway

There are several known pathways that affect aging in *C. elegans* (i.e., the insulin pathway, the dietary restriction system, and the mitochondrial respiration system) (Amrit, *et al.* 2014, Collins, *et al.* 2008). Here, we performed genetic analyses to determine whether and which of the three known aging pathways might be associated with the prolongevity effect of Astaxanthin. We reasoned that animals with mutations in pathways affected by Astaxanthin would show reduced...
effects in the presence of the carotenoid on the lifespan extension. Among of the longevity-associated pathway in *C. elegans* is the DAF-16/DAF-2 insulin pathway (Lin et al. 2001). We noticed that similarly to the wild-type animals, Astaxanthin caused an (additional) increase in the lifespan of the *daf-2* (e1370) (P-value = 0.001) and *daf-16* (mu86) (P-value = 0.000046) mutant animals (Fig. 2). Similarly, feeding *C. elegans* glp-1 (or178) mutants (Libina et al. 2003, TeKippe and Aballay 2010) with Astaxanthin resulted in additional extension of the lifespan (P-value = 0.000024, Fig. 2). We therefore concluded that Astaxanthin effect on *C. elegans* lifespan are independent of the insulin/IGF signaling pathway. Another well-characterized pathway of lifespan extension involves dietary restriction (Lakowski and Hekimi 1998). Here too, feeding *eat-2* (ad1116) mutant animals with Astaxanthin resulted with an extended lifespan (P-value = 0.011, Fig. 2), indicating that Astaxanthin effect on *C. elegans* lifespan are also independent of the dietary restriction mechanism.

In addition to the insulin/IGF signaling and dietary restriction pathways, life-extending in animals also involves the mitochondrial respiration system (i.e., mitochondrial dysfunctions resulting with extended lifespan) (Murakami and Murakami 2005). It is postulated that mutations in genes encoding components of the mitochondrial electron transport chain (ETC) lower the levels of ROS and thereby may increase the lifespan of the animals (Murakami and Murakami 2005). Electron leakage from complex III is suggested to be a major contributor to the generation of ROS within the mitochondria (Brand 2010, Chen et al. 2003, Quinlan et al. 2013). To test whether reduced respiration activities may influence the prolongevity-mediated effects by Astaxanthin, we analyzed the lifespan of *C. elegans* mutants in genes encoding respiratory-related proteins. Mutants in the Rieske iron-sulfur protein (RISK) of complex III (i.e., *isp-1* mutants, also denoted as *qm150*), showed reduced (shortened) lifespan when they were treated (fed) with Astaxanthin (P-value = 0.000011, Fig. 2). These results strongly suggest that the Astaxanthin-mediated longevity effects involve complex III. The effects of Astaxanthin on the lifespan was further analyzed in *C. elegans* mutants affected in other mitochondrial proteins. *CLK-1* encodes a demethoxyubiquinone hydroxylase enzyme required for ubiquinone biosynthesis. *C. elegans* *clk-1* mutants show a pleiotropic phenotype that includes slowed development and aging (Branicky et al. 2006). However, the data showed that Astaxanthin-mediated longevity was not affected in the *clk-1* (e2519) animals (P-value = 1.1 x 10^{-7}, Fig. 2). Feeding *C. elegans* *gas-1* (fc21) (affected in complex I) or *mev-1* (kn1) (affected in complex II) mutants with Astaxanthin, resulted with mild extensions in longevity (P-values 0.0024 and 0.2831, respectively). Taken together, these data indicate that the Astaxanthin-extended
lifespan effects we see in *C. elegans* rely on the level or activity of respiratory complex III, and maybe to some extend on other respiratory-mediated functions.

**Astaxanthin affects the biogenesis of the native respiratory complex III, *in vitro***

The Astaxanthin-mediated effects we see suggest that Astaxanthin increases the lifespan of *C. elegans* by influencing the biogenesis or function of the respiratory system via CIII and maybe CI and CII as well (Fig. 2). To analyze whether Astaxanthin directly affect the biogenesis or activities of these respiratory complexes, we used *in vitro* analyses with crude mitochondria preparations (i.e., ‘in-organello’ assays), obtained from both animals and plants. These include *C. elegans*, rodents, human and cauliflower mitochondria (*Brassica oleracea* var. botrytis) (see Materials and Methods). For this purpose, Astaxanthin was extracted from the algae *Haematococcus pluvialis*, solubilized in 5% DMSO and then incubated with the mitochondria for 30 min on ice. The integrity of native respiratory complexes in *C. elegans*, cauliflower, rodents and human, was investigated by BN-PAGE analyses (Fig. 3 and Fig. S5).

Figure 3A shows the effects of increased Astaxanthin concentrations (i.e., 0, 9 and 14 μM) on native respiratory complexes in animal mitochondria. A decreased level of a high-molecular mass band (about 500 kDa), which we expected to corresponds to a native complex III dimer (i.e., holo-CIII2), was observed in *C. elegans* mitochondria treated with Astaxanthin. Notably, this Astaxanthin-mediated reduction in the ~500 kDa band, was followed by the appearance of a ~350 kDa band, which may corresponds to a partially disassembled CIII particle (i.e., CIII*). BN-PAGEs of crude mitochondria preparations obtained from rodents and human treated Astaxanthin showed the same patterns (i.e., reduced intensities of the ~500 kDa complex and an accumulation of ~350 kDa particles) (Fig. 3A). To analyze whether similar effects may occurs in vivo, we analyzed the integrity of respiratory complexes in *C. elegans* fed with Astaxanthin. For this purpose, young adult worms were grown for 7 days in the presence of wild-type *P. marcusii*, washed several times to remove bacterial contaminants and crude mitochondria preparations were obtained, essentially as described previously (Grad, *et al.* 2007). Organellar proteins from *E.coli* (-Asta) and *P. marcusii* (+Asta) fed *C. elegans* were separated by under native conditions. The BN-PAGE assays indicated that the absorption or uptake of Astaxanthin (Fig. S4) also resulted with a reduction in the 500 kDa band and the accumulation of the 350 kDa particles (Fig. S5).

We next analyze the effects of Astaxanthin on mitochondria isolated from cauliflower inflorescences, as this tissue allows the purification of large quantities of highly-pure mitochondria preparations (*Keren, et al.* 2009, Neuwirt, *et al.* 2005, Sultan, *et al.* 2016).
Mitochondria respiratory complexes were then separated by BN-PAGE analysis (Fig. 3B and Supplementary Fig. S6). Arrows indicate to native respiratory complexes CI (~1,000 kDa), a CIII dimer, (CIII$_2$, about 500 kDa), CIV (~220 kDa) and CV (~600 kDa) in cauliflower mitochondria. In plant’s mitochondria as well, the addition of Astaxanthin led to a notable reduction in the levels of the 500 kDa corresponding to CIII$_2$, followed by the accumulation of a lower (about 300 kDa band) corresponding to sub-CIII particle (CIII*) (Fig. 3B). These were correlated with the levels of Astaxanthin added to the isolated mitochondria (Fig. 3C). BN-PAGEs followed by immunoblot analyses with antibodies raised to RISP (iron-sulfur protein of complex III), indicated that both the ~350 kDa and 500 kDa particles contain RISP (Fig. 3 and Fig. S6), strongly supporting that these particles correspond to holo-CIII$_2$ and sub-CIII*. The Astaxanthin-mediated effects (i.e., decreased CIII$_2$ levels and increased amounts of the 350 kDa band) seem specific to Astaxanthin, as mitochondria treated with DMSO alone had no obvious effects on the levels or integrity of respiratory complexes I, III and V in cauliflower mitochondria (Fig. 3B), while the addition of β-Carotene or Zeaxanthin had no obvious effects on the integrity of the organellar complexes (Fig. S5B). Mass-spectrometry (LC-MS/MS) analyses of gel slices corresponding to holo-CIII$_2$ (upper band) and CIII* particles (lower band) obtained from human (Table S3) and plant (Table S4) mitochondria, indicated the presence of numerous subunits of CIII (see Table 2). The LC-MS/MS data also indicated the presence of many other peptides that are corresponding to CI (mainly), CIII, CIV, CV and various other known mitochondrial proteins. These may be associated with the CIII particles. However, it is anticipated that these proteins rather correspond to various sub-particles of respiratory complexes that are migrating with similar masses to those of the holo-CIII$_2$ and CIII*, as only the immunoblots with RISP analyses indicated a correlation with the reduction in the CIII dimer and to increased levels of the lower (350 kDa) band (Figs. 3C and S6). As indicated by immunoblots, the presence of RISP protein in the holo-CIII$_2$ and CIII* of both human and cauliflower mitochondria, was also supported by the LC-MS/MS data (Table 2 and Supplementary Tables S3 and S4).

In addition to CIII, Astaxanthin may also affects the accumulation of CI (using anti-CA2 antibodies) and the ATP-synthase (CV, anti-ATPA antibodies) in cauliflower mitochondria (Fig. 3C and Fig. S6). BN-PAGE analyses followed by immunoblots corresponding to CI (with anti-CA2 antibodies) and ATP-synthase (CV, anti-ATPA antibodies) indicated to reduced holo-CI (~1,000 kDa) and a ~1,500 kDa particle, corresponding to a ‘supercomplex’ containing CI and CIII$_2$ (i.e., CI+CIII$_2$), which was also apparent in the
immunoblots with anti-RISP antibodies (Figs. 3C and S6). Yet, the pigment has no obvious effect on the levels and biogenesis of the native complex V (Fig. 3C and Fig. S6).

The steady-state levels of various mitochondria proteins in Astaxanthin-fed *C. elegans* was assayed by immunoblot analyses, with antibodies generated to various respiratory complexes, including CI (C25H3.9), CIII (ISP-1), CIV (CTC-1) and CV (ATP-5) and cytochrome C (CYC-1) (see Table S2). In each assay, the steady-state levels of different proteins in the Astaxanthin-fed animals were compared to those of untreated wild-type animals of the same age at different total protein loadings (i.e., 0.15 up to 1.5 times) (Fig. 4). The levels of various mitochondrial proteins, including C25H3.9 (CI), CTC-1 (CIV) and ATP-5 (CV) did not changed significantly between the control and Astaxanthin-fed animals or the *C. elegans isp-1* mutants (Fig. 4B). A reduction in the signal of cytochrome C (CYC-1) was observed in *C. elegans isp-1* mutants, but not in animals fed with Astaxanthin. We also noticed a notable reduction in RISP signals in both *isp-1* animals and wild-type *C. elegans* fed with Astaxanthin. While a reduction in RISP is expected in the case of the *isp-1* mutant-line (Fig. 4, ISP-1 panel), it remains unclear why a notable reduction in RISP is seen in animals fed with Astaxanthin, whereas the protein remains intact in the ‘in organello’ assays. A reasonable explanation is that when the worms are fed with Astaxanthin they undergo continues disassembly of CIII (or the supercomplex of CI-CIII). Under these conditions, various complex III (and possibly CI) subunits, including the RISP protein, may become more susceptible to proteolysis by organellar proteases and peptidases, while much lower degradation rates are expected under the in vitro conditions (i.e., short incubations on ice).

*C. elegans* treated with Astaxanthin display altered respiratory functions

Analyses of native mitochondrial complexes, using denaturing PAGE profiles, suggested to alter CIII and maybe CI biogenesis in *C. elegans* fed with Astaxanthin. To determine whether the respiratory activity was affected in animals fed with Astaxanthin, we monitored the oxygen (O$_2$) uptake rates of non-treated versus Astaxanthin-fed wild-type *C. elegans* and *isp-1* mutants, using a Clark-type electrode (Hansatech Instruments, Norfolk, UK). For this purpose, synchronized late L4 worms were collected and washed several times with M9 buffer. Oxygen consumption was monitored in the worms in a sealed chamber (see e.g., (Cohen et al. 2014, Keren, et al. 2012, Shevtsov, et al. 2018, Zmudjak et al. 2013). Each respiration chamber contained 250 µl of packed *C. elegans* suspension diluted 10 times in M9 buffer. These measurements indicated that *C. elegans* fed on Astaxanthin and the *isp-1* mutants have reduced respiration activities (i.e., $15.03 \pm 3.03$ and $14.33 \pm 2.88$ nmol O$_2$-min$^{-1}$-mg$^{-1}$ total protein),
respectively, compare to those of the non-treated wild-type animals (i.e., 28.83 ± 3.07 nmol O₂·min⁻¹·mg⁻¹ total protein) (Fig. 4).

Here, we also measured the O₂-uptake rates in the presence of various inhibitors, including Rotenone (Rot), which inhibits complex I electron transport, the complex III inhibitor Antimycin A (Anti A) and Potassium cyanide (KCN) that blocks electron transport from complex IV to oxygen. As shown in Figure 4, both Rot and Anti-A have noticeable effects on the respiratory activities of the non-treated wild-type, compared to those of isp-l mutant or C. elegans fed with Astaxanthin (55% ± 2.95 and 66% ± 0.70 compared to 34% ± 0.99 O₂·min⁻¹·mg⁻¹ total protein, respectively). In contrast, KCN had similar effects on the O₂-uptake rates of wild-type worms, worms fed on Astaxanthin and the isp-1 mutants (85% ± 5.18 and 90% ± 2.35, and 95% ± 0.19, O₂·min⁻¹·mg⁻¹ total protein, respectively) (Fig. 4). These results further indicate to altered respiratory CI- and CIII-mediated activities.

**Astaxanthin inhibits enzymatic activities of mammalian complexes I and III.**
The BN-PAGE (Fig. 3 and Suppl. Figs. S4-S6), LC-MS/MS (Table S4) and the respiration activity data (Fig. 4) indicate that Astaxanthin has a strong effect on the biogenesis and functions of CIII, and presumably affects CI and the supercomplex I+III, as well. These data were further supported by analyses of the enzymatic activities of native respiratory complexes in the presence or absence of Astaxanthin. Table 1 shows the effects on the respiratory functions of mitochondrial complexes isolated from mouse heart that were pre-incubated with DMSO alone (Mock) or with purified Astaxanthin dissolved in DMSO (see Materials and Methods). The biochemical assays indicated to significant decreases in the activities of both complexes I and III. The inhibitory effect of Astaxanthin was even more pronounced in enzymatic activity assays of CI+CIII, which approached the respiratory assay sensitivity limits. These analyses also indicated that complexes II, IV and citrates synthase enzyme where not, or only slightly, affected by the addition of Astaxanthin to animals mitochondria.

**Discussion**
The nematode *Caenorhabditis elegans* is a well-established animal system to study organismal aging, and for the characterizing and identifying new drugs that can extend lifespan and improve the quality of old age in animals. Here, we used *C. elegans* to study the molecular basis of the anti-aging effects of Astaxanthin consumed by various organisms. Our results show that Astaxanthin extends *C. elegans* lifespan by 20%-40%. The effects on the life span seem specific
to Astaxanthin, as other carotenoids tested here, including Zeaxanthin and Lycopene had no lifespan extension effects. In addition, the anti-aging role of Astaxanthin had no obvious effects on the reproduction, age pigmentation and locomotion of the animals. The life-extension phenotype, induced by Astaxanthin, relies mainly on isp-1 mutants that are affected in mitochondria respiratory complex III, but not on gas-1 (complex I) and clk-1 (ubiquinol biosynthesis), nor on other aging-related pathways (i.e., the insulin/IGF-1 and dietary restriction pathways), as indicated by lifespan measurements of daf-2/daf-16, eat-2 or glp-1 mutants fed with Astaxanthin.

Remarkably, Astaxanthin added to isolated mitochondria causes a reduction in the levels of holo-complex-III$_2$ and supercomplex I+III, and to the appearance of lower molecular-mass particles that correspond to sub-CIII particles. Accordingly, BN-PAGE and LC-MS/MS analyses indicated the presence of various CIII subunits in both the high and lower mass particles. The presence of various complex I subunits in these gel-bands, may relate to the strong association of complexes I and III, which together with complex IV also form a stable supercomplex known as the Respirasome (I$_1$I$_1$I$_2$IV$_1$) (Schafer et al. 2006). Alternatively, it remains possible that sub-complex I, IV and V (natively formed or disassemble during mitochondria preparations or gel-run) may migrate with similar masses under the experimental conditions. Together, these analyses indicate that Astaxanthin affects the assembly or biogenesis of complex III and the CI-CIII$_2$ supercomplex, whose functional roles and assembly are under investigation (Signes and Fernandez-Vizarra 2018). As CIII biogenesis / disassembly are observed in both animal and plant mitochondria (Figs. 3, S5 and S6), the data imply to a universal effect caused by Astaxanthin to CIII, in all eukaryotes.

Reduced accumulation of CIII is expected to affect the respiratory functions of C. elegans cells fed with Astaxanthin. The O$_2$-uptake rates of worms fed on Astaxanthin or isp-1 mutants were reduced by about 2x folds (Fig. 5), compared to those measured in the non-treated (control) C. elegans. Similarly, the oxygen consumption of Astaxanthin-treated mitochondria, isolated from mouse cells was lowered by more than 50%, and to reduced activities of the respiratory complexes I and III. Currently, it remains unclear how Astaxanthin affects the assembly or biogenesis of complex III in plants and animals. It is possible that Astaxanthin interacts with complex III and thereby interfere with the assembly process or destabilizes the integrity of the native complex. Due to its skeletal properties (long polyene chain as well as two oxygenated β-ionone-type rings), Astaxanthin seems to share some chemical equivalents with ubiquinol (contains a single benzylquinone head-group ring). Astaxanthin may therefore interfere with the association of ubiquinol with CIII, affecting RISP stability, the assembly state
of the holo-CIII or the integrity of the CI-CIII₂ suppercomplex. Another possible effect of Astaxanthin may involve cardiolipin, a unique phospholipid that is an important component of the inner-mitochondrial membrane. Crystal structures show that cardiolipin (CL) is tightly associated with CIII in vivo (Lange et al. 2001) and affects the assembly of various respiratory complexes (Paradies et al. 2014). The absorption of Astaxanthin (in vivo or in vitro) may interfere with the association of CL with CIII and thereby affects its biogenesis and function. However, such speculations require further investigation.

At this point, it is also not clear to us why RISP was reduced in C. elegans fed with Astaxanthin, but seem to remained intact in the ‘in organello’ assays of mitochondria treated with Astaxanthin (Figs. 3 and S4). The data indicate that Astaxanthin affects the assembly of CIII, as evident by the appearance of lower molecular mass particles corresponding to sub-CIII particles in the mitochondria. Disassembly of CIII is expected to affect the stability of various CIII subunits, including RISP, leading to their degradation over time, whereas under in organello assays (i.e., short incubations on ice; see Materials and Methods), RISP (or various other CIII subunits) remain intact and are less prone for degradation by the organellar proteases.

Mitochondria are major sites for energy metabolism and ATP production in eukaryotic cells, through the oxidative phosphorylation pathway. In light of the expected significance of mitochondria functions to animal and plant physiology, why does the reduced CIII activity and level in Astaxanthin-fed animals or the isp-1 mutants results with an extended life span? Currently, we cannot provide a definitive explanation, but we speculate that these phenotypes relate to reduced mtROS production. ROS are known to induce oxidative damages of fatty acyl chains of membrane lipids, by ‘lipid-peroxidation’ (Tsuchiya et al. 1994). Altered membrane integrity has many pathological consequences in human, such as neurological diseases, inflammations, diabetes, cancer, and aging. Astaxanthin is a strong antioxidant that was recently suggested to reduce mtROS protection in C. elegans and other animals (Kuraji et al. 2016, Liu, et al. 2016, Wu et al. 2015). Our data indicate to an additional mechanism by which Astaxanthin affects the life span of animals. The association of Astaxanthin with CIII destabilizes the native complex, resulting with lower molecular mass CIII intermediates and reduced respiration. Lowered respiration activities are likely associated with reduced rates of ‘toxic’ electron leakage from complex III, a major contributor to the generation of mtROS (Brand 2010, Chen et al. 2003, Quinlan et al. 2013), and thereby can protect the cells (and organelles) from oxidative damages. Accordingly, the aging related mechanism through mitochondria activities have been previously related to ROS production (Murakami and Murakami 2005, Quinlan, et al. 2013).
In summary, our data provide with novel insights into the molecular mechanism of longevity extension mediated by Astaxanthin in *C. elegans*. Biochemical and genetic studies indicate that Astaxanthin affects complex III biogenesis and activity, and may increases longevity by protecting the animal cells against ROS and oxidative stresses, due to complex III-associated ‘electron leakage’ during respiration. These effects may be universal among different eukaryotes, as similar effects on CIII biogenesis or assembly were noted in plants, warms, rats and human. Our data may also become useful for the development of multi-target drugs designed to CIII in order to prolong the lifespan of animals.

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Table 1. The effects of Astaxanthin on the enzymatic activities of mouse heart mitochondrial respiratory complexes.

| Assay                                      | Activity<sup>1</sup> |   |   |
|--------------------------------------------|-----------------------|---|---|
|                                            | Vehicle (DMSO<sup>2</sup>) | Astaxanthin<sup>3</sup> | residual activity |
| NADH-CoQ reductase (CI)                    | 354                   | 142 | 40% |
| NADH-cytochrome c reductase (CI+III)       | 1464                  | 78  | 5.3% |
| Succinate dehydrogenase (CII)              | 498                   | 513 | 103% |
| Ubiquinone- cytochrome c reductase (CIII)  | 4160                  | 1781| 43% |
| Cytochrome c oxidase (CIV)                 | 3638                  | 2828| 77% |
| Citrate synthase                           | 1376                  | 1402| 101% |

<sup>1</sup> - Enzyme activity (nmol O<sub>2</sub> · min<sup>-1</sup> · mg<sup>-1</sup>)

<sup>2</sup> - 5% (v/v) DMSO

<sup>3</sup> - 10 µM Astaxanthin
Table 2. Summary of mass-spectrometry (MS) of the ~500 kDa (higher) and ~350 kDa (lower) particles in animals (human) and plant (cauliflower) mitochondria, treated with Astaxanthin.

| Associated respiratory complex | Human | Cauliflower |
|-------------------------------|-------|-------------|
|                               | No. of subunits identified in the 500 kDa band | No. of subunits identified in the 350 kDa band | No. of subunits identified in the 500 kDa band | No. of subunits identified in the 350 kDa band |
| CI (NADH-dehydrogenase)       | 40    | 20          | 11    | 9           |
| CII (succinate dehydrogenase) | 2     | 2           | 2     | 2           |
| CIII (Cytochrome c reductase) | 13    | 12          | 11    | 10          |
| Rieske iron-sulfur protein    | +     | +           | +     | +           |
| CIV (Cytochrome c oxidase)    | 15    | 11          | 1     | 1           |
| CV (ATP-synthase)             | 13    | 11          | 8     | 7           |
Figure legends

Figure 1. The effects of Astaxanthin on the lifespan of C. elegans.
Survival curves of C. elegans fed with Astaxanthin. Between 70-80 animals were used in each experiment. (A). Animals were fed with wild-type Paracoccus marcusii (produce Astaxanthin, green), P. marcusii ΔcrtB mutant (lacks carotenoids, orange) or E. coli (OP50) cells (blue). The results are the average of 4 independent experiments. (B). C. elegans fed with E. coli-pASTA (expressing Astaxanthin, green) and E. coli cells containing an empty vector (E.coli pACYC184, orange). The mean lifespan (in days) was 19.97 ± 0.74, compared to 17.34 ± 0.69 in the control cells. The results are the average of 5 independent experiments. (C). Animals fed with purified Astaxanthin solubilized with DMSO (green), E. coli (OP50) cells (orange) and E. coli (OP50) cells + DMSO (blue). The results are the average of 5 independent experiments. (D). The half-life’s of worms fed with P. marcusii (produce Astaxanthin, green) and P. marcusii ΔcrtB mutant (lacks carotenoids, orange). The results are the average of 7 independent experiments. The values are means of five biological replicates (error bars indicate one standard deviation). Asterisk in panel D indicates a significant difference from control (Student’s T-test, P 0.05). Lifespan curves were analyzed by plotting Kaplan-Meier survival curves (Goel, et al. 2010). Mean lifespan data was compared using Log-rank test (Mantel 1966) with appropriate correction for multiple comparisons OASIS-2.

Figure 2. The effects of Astaxanthin on the longevity of C.elegans mutated in different life-extending genes
Survival curves of nematodes mutated in genes that regulate lifespan through different pathways supplemented with Astaxanthin. Worms mutated in the daf-2(e1370), daf-16(mu86), eat-2(ad1116), glp-1(or178), isp-1(qm150), mev-1(kn1), gas-1(fc21) or clk-1(e2519) were fed with P. marcusii (produce Astaxanthin, red), P. marcusii ΔcrtB mutant (lacks carotenoids, blue). Nematode survival was calculated with the Kaplan-Meier method (Goel, et al. 2010), and differences in survival rates were tested for significance using the log-rank test (Mantel 1966), with appropriate correction for multiple comparisons OASIS-2.

Figure 3. The Effect of Astaxanthin on isolated plant mitochondria.
(A). Astaxanthin was added at different concentrations (i.e., 0, 9 and 14 μM) to crude mitochondria preparations, obtained from C. elegans, human and mice. Native organellar complexes were separated by BN-PAGE, followed by Coomassie Blue staining. The position
of complex III, which partially disassembled in the presence of Astaxanthin, is indicated by arrows. (B). The effects of Astaxanthin on various respiratory complexes in cauliflower mitochondria was analyzed by BN-PAGE assays. (C) Increasing amounts of Astaxanthin (as indicated in the gel) were added to isolated mitochondria of cauliflower. Arrows indicate to native complexes I, III and V. The effects of Astaxanthin on CIII was analyzed by western-blot analysis with antibodies raised against plant-specific RISP (Rieske iron sulfur) protein. The blots indicate that Astaxanthin cause a reduction in the levels of the holo-complex III and supercomplex I+III, and to the appearance of protein-bands of lower molecular-masses.

**Figure 4. Changes in the mitochondria RISC complex of C. elegans grown on bacteria expressing Astaxanthin.** (A). Western-blot analysis of crude mitochondria obtained from wild-type C. elegans at different concentrations, C. elegans fed with Astaxanthin (+Asta), and C. elegans isp-1 mutant-line. Antibodies against NUFB5 (a subunit of the mitochondrial NADH dehydrogenase (ubiquinol) of the mitochondrial complex I (ortholog in C. elegans is termed as C25H3.9), Rieske iron sulphur protein (ISP-1) subunit of the mitochondrial complex III, COX1 (cytochrome c oxidase subunit 1) of the mitochondrial complex IV (the ortholog in C. elegans is termed as CTC-1), ATP5A (ATP synthase subunit alpha subunit) of mitochondrial complex V (the orthologue in C. elegans is ATP-5), and Cyt C (cytochrome c reductase; the ortholog in C. elegans is CYC-1), were used in immunoblotting assays. (B). SDS-PAGE followed by Coomassie staining of isolated mitochondria from C. elegans fed with Astaxanthin (+Asta). Controls included crude mitochondria isolated from wild-type C. elegans that were not fed with Astaxanthin, and mitochondria isolated from the C. elegans isp-1 mutant-line.

**Figure 5. Respiration activities in C. elegans wild-type and isp-1 mutants fed with Astaxanthin.** O₂-uptake rates of wild-type, Astaxanthin-fed animals and isp-1 mutants were analyzed with a Clark-type electrode as described previously (Cohen, et al. 2014). For each assay, equal amounts of dense packed worms from 5 different plates were submerged in 2.25 mL M9 buffer and applied to the electrode in a sealed glass chamber in the dark. O₂-uptake rates were measured in the absence (Control) or in presence of rotenone (+ROT, 50 μM), Antimycin A (Anti A, 50 μM) and KCN (1 mM) which inhibit complexes I, III and IV activities (respectively). The values are means of four biological replicates. Error bars indicate one standard deviation.
Supplementary data

Table S1. Carotenoid composition in bacterial strains used in this work.

Table S2. List of antibodies used for the analysis of astaxanthin effects on C. elegans lifespan.

Table S3. Mass-spectrometry (MS) of the ~500 kDa (higher) and ~350 kDa (lower) particles in human mitochondria treated with Astaxanthin. Peptides highlighted with orange color indicate to CI subunits, in blue CII subunits, in red CIII subunits, in green CIV subunits, in yellow CV (i.e., ATP-synthase).

Table S4. Mass-spectrometry (MS) of the ~500 kDa (higher) and ~350 kDa (lower) particles in cauliflower mitochondria treated with Astaxanthin. Peptides highlighted with orange color indicate to CI subunits, in blue CII subunits, in red CIII subunits, in green CIV subunits, in yellow CV (i.e., ATP-synthase).

Figure S1. Chemical structures of the carotenoids Zeaxanthin and Astaxanthin compared with ubiquinone.
Molecular structures of Zeaxanthin and Astaxanthin as well as ubiquinone.

Figure S2. Lifespan of C. elegans fed with Zeaxanthin.
A survival curve of nematodes supplemented with zeaxanthin in different stages of the development. About 75 animals were fed with P. marcusii bacteria that produce zeaxanthin (red), control P. marcusii bacteria (blue) or control E. coli OP50 bacteria (green). Animal survival was calculated with the Kaplan-Meier method, and survival differences were tested for significance using the log-rank test compared with the control. The mean lifespans (in days) of the different groups were 18.32 ± 0.53, 23.43 ± 0.57 and 16.29 ± 0.73, respectively. Lifespan curves were analyzed by plotting Kaplan-Meier survival curves (Goel, et al. 2010). Mean lifespan data was compared using Log-rank test (Mantel 1966) with appropriate correction for multiple comparisons OASIS-2.

Figure S3. Astaxanthin effect on the median lifespan at the adult stage.
A survival curve of nematodes supplemented with Astaxanthin in different stages of the development. About 75 animals were used in the experiment. Worms were fed with Paracoccus marcusii with no carotenoids expression from the embryo stage until L4 stage, and from L4 stage until their death with P. marcusii which produce Astaxanthin (blue), another group of
worms were fed from the embryo stage until L4 stage with P. marcusii which produce Astaxanthin, and from L4 stage until their death with P.M. with no carotenoids expression (red). The two control groups included (i) worms which were fed from the embryo stage until their death, with *P. marcusii* with Astaxanthin (green); (ii) worms which were fed from the embryo stage until their death with *P. marcusii* with no carotenoids expression (purple). *C. elegans* survival was calculated with the Kaplan-Meier method, and survival differences were tested for significance using the log rank test compared to the control. The mean lifespans (in days) of the groups given *P. marcusii* with no carotenoids expression during development and *P. marcusii* with Astaxanthin during adulthood, *P. marcusii* with Astaxanthin during development and *P. marcusii* with no carotenoids expression during adulthood, *P. marcusii* with Astaxanthin during all their lives and *P. marcusii* with no carotenoids expression during all their lives were 19.87 ± 0.48, 17.95 ± 0.57, 20.18 ± 0.55 and 18.22 ± 0.49, respectively. Lifespan curves were analyzed by plotting Kaplan-Meier survival curves (Goel, *et al.* 2010). Mean lifespan data was compared using Log-rank test (Mantel 1966) with appropriate correction for multiple comparisons OASIS-2.

**Figure S4. Astaxanthin from *P. marcusii* is absorbed by *C. elegans* cells.**

(A). Stereo microscope photograph of Astaxanthin-fed animals. *C. elegans* fed with *P. marcusii* expressing Astaxanthin from the embryo stage for 5 days (left panel). After 5 days the worms were fed with *E. coli* bacteria for 24 hours, in order to wash their intestines (middle panel). The control worms were fed just with *E. coli* bacteria (right panel). (B). Quantification of Astaxanthin in *P. marcusii* and in *E. coli* cells by HPLC. The amount of Astaxanthin is shown as microgram per gram dry weight (µg/gDW). Stereomicroscope images of Astaxanthin-fed animals. *C. elegans* were fed with *P. marcusii* containing Astaxanthin (left and middle panels) or control *P. marcusii* (left panel) from the embryo stage until adulthood. Then one group of worms were fed with *E. coli* for 24 hours, which cleans their intestine (middle panel).

**Figure S5. Accumulation of respiratory complexes in *C. elegans* mitochondria treated with astaxanthin.**

(A). Crude mitochondria were obtained from *C. elegans* grown in the absence (-) and presence of *P. marcusii*. Mitochondrial complexes were separated by BN-PAGE. (B). Crude mitochondria obtained from *C. elegans* were treated in absence (-) or presence (+) of Astaxanthin (10 µM), Lycopene (5 and 10 µM), and Zeaxanthin (5 and 10 µM), and then
separated by BN-PAGE. The positions of the holo-complex III dimer (CIII₂) and sub-CIII (CIII*) particles are indicated by arrows.

**Figure S6. Accumulation of respiratory complexes in cauliflower mitochondria treated with different concentrations of Astaxanthin.**

Astaxanthin, at various concentrations (as indicated in the blots), was added to cauliflower mitochondria. The mitochondrial complexes were then separated by BN-PAGE, and the gel was stained by Coomassie brilliant blue. Antibodies raised against CA2 (Carbonic anhydrase subunit 2) and ATPA (ATP synthase subunit alpha) were used to assay the levels and integrity of the organellar complexes. The positions of the native CI (~1,000 kDa), CV (about 600 kDa) and supercomplex I+III are indicated.
Figure 1

A

B

C

D

**Figure 1.**

A: Graph showing percent survival over time (days) for different groups. The graph compares the survival rates of different bacterial strains and the addition of astaxanthin from *P. marcusii*.

B: Graph showing percent survival over time (days) for different groups. The graph compares the survival rates of different bacterial strains and the addition of astaxanthin from *P. marcusii*.

C: Graph showing percent survival over time (days) for different groups. The graph compares the survival rates of different bacterial strains and the addition of astaxanthin from *P. marcusii*.

D: Bar graph showing age in days at 50% mortality for different conditions. The graph compares the effect of astaxanthin from *P. marcusii* on the age at 50% mortality of bacteria.
Figure 2

P. marcusii

P. marcusii ΔcrtB
Figure 3

A. Coomassie Immunoblots with anti-RISP from C. elegans, Human, and Mice mitochondria treated with Astaxanthin. Arrowheads indicate the presence of CI + III+2 (CIII*).

B. Cauliflower mitochondria treated with Astaxanthin (Asta) and DMSO. Arrowheads indicate the presence of CI + III+2 (CIII*) in Coomassie staining.

C. Cauliflower mitochondria treated with Astaxanthin (Asta) and DMSO. Arrowheads indicate the presence of various complexes in Coomassie and Immunoblot staining.
### Figure 4

#### A

|          | x0.15 | x0.75 | x0.5 | x1.0 | x1.5 | +Asta | -Asta |
|----------|-------|-------|------|------|------|-------|-------|
| **CI** (C25H3.9) |       |       |      |      |      |       |       |
| **CIII** (ISP-1)   |       |       |      |      |      |       |       |
| **CIV** (CTC-1)    |       |       |      |      |      |       |       |
| **CV** (ATP-5)     |       |       |      |      |      |       |       |
| **CYC-1**          |       |       |      |      |      |       |       |

- **CI** protein: 17 kD
- **CIII** protein: 25 kD
- **CIV** protein: 57 kD
- **CV** protein: 55 kD
- **CYC-1** protein: 17 kD

#### B

|          | x0.15 | x0.75 | x0.5 | x1.0 | x1.5 | +Asta | -Asta |
|----------|-------|-------|------|------|------|-------|-------|
| **Mw (kDa)** |       |       |      |      |      |       |       |

- **CI** protein: 17 kD
- **CIII** protein: 25 kD
- **CIV** protein: 57 kD
- **CV** protein: 55 kD
- **CYC-1** protein: 17 kD

**Note:** Mw (Molecular Weight) in kDa represents the molecular weight in kilodaltons.
|       | Respiration activity (nmol O₂ min⁻¹ mg⁻¹ protein) | % of inhibition |
|-------|-----------------------------------------------|-----------------|
|       | Respiration activity (nmol O₂ min⁻¹ mg⁻¹ protein) | ROT (50 μM)     | Anti-A          | KCN (1 mM) |
| WT    | 28.83 ± 3.07                                  | 55.17 ± 2.95    | 66.17 ± 0.70    | 85.71 ± 5.18 |
| isp-1 | 14.33 ± 2.88                                  | 34.17 ± 1.33    | 34.71 ± 0.99    | 95.22 ± 0.19 |
| astaxanthin | 15.03 ± 3.03                            | 39.09 ± 1.44    | 40.20 ± 0.84    | 90.05 ± 2.35 |

Figure 5
Supplementary Figure S1. Chemical structures of the carotenoids Zeaxanthin and Astaxanthin compared with ubiquinone.
Supplementary Figure S2. Lifespan of *C. elegans* fed with Zeaxanthin.
Supplementary Figure S3. Astaxanthin effect on the median lifespan at the adult stage.
**Supplementary Figure S4. Astaxanthin from P. marcusii is absorbed by C. elegans cells.**

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**B. HPLC quantitative analysis of astaxanthin in bacteria**

| Sample            | Mass of sample (mg) | Amount of astaxanthin (μg/gDW) |
|-------------------|---------------------|---------------------------------|
| E.coli+ Asta      | 0.311               | 146.3066                        |
| P. marcusii+ Asta| 0.422               | 715.9167                        |

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Supplemental Figure S5. Accumulation of respiratory complexes in *C. elegans* mitochondria treated with Astaxanthin, Lycopene and Zeaxanthin.
Supplemental Figure S6. Accumulation of respiratory complexes in cauliflower mitochondria treated with different concentrations of astaxanthin.
### Supplementary Table S1. Carotenoid composition in bacterial strains used in this work

| Name                                           | Carotenoid composition                                      |
|------------------------------------------------|-------------------------------------------------------------|
| *Escherichia coli* WT                          | None                                                        |
| *E. coli* pASTA                                | Astaxanthin                                                 |
| *E. coli* pACYC184                             | None                                                        |
| *Paracoccus marcusii* (P.maricusii) wild-type   | Largely Astaxanthin                                         |
|                                                | Minor: Phoenicoxanthin Adonixanthin, 3'-Hydroxyechinenone   |
| *P. marcusii* ΔcrtB                            | None                                                        |
| *P. marcusii* ΔcrtW                            | Zeaxanthin                                                  |
| *P. marcusii* ΔcrtY                            | Lycopene                                                    |
Supplementary Table S2. List of antibodies used for the analysis of astaxanthin effects on *C. elegans* lifespan.

| Antibody | Protein I.D. | origin | serum | dilution | Reference / source |
|----------|--------------|--------|-------|----------|--------------------|
| Atp-A    | Mitochondrial ATP-synthase subunit α | Zea mays | Mouse (monoclonal) | 1/5,000 | (Michael *et al.* 1993) |
| ATP-5    | ATP synthase subunit, mitochondrial | *Caenorhabditis elegans* | Rabbit (polyclonal) | 1/1,000 | Thermo Fisher Scientific |
| C25H3    | Uncharacterized protein, mitochondrial respiratory complex I | *Caenorhabditis elegans* | Rabbit (polyclonal) | 1/1,000 | Sigma-Aldrich Co. LLC |
| CA2      | γ-carboxic anhydrase-like subunit 2 | *Arabidopsis thaliana* | Rabbit (polyclonal) | 1/1,000 | (Perales *et al.* 2005, Sunderhaus *et al.* 2006) |
| CTC-1    | Cytochrome c oxidase subunit 1, complex IV, mitochondrial | *Caenorhabditis elegans* | Mouse (monoclonal) | 1/1,000 | Thermo Fisher Scientific |
| CYC-1    | Cytochrome C, mitochondrial | *Caenorhabditis elegans* | Rabbit (polyclonal) | 1/1,000 | Schagger H.H. *et al.* (2002) |
| ISP-1    | Cytochrome b-c1 complex subunit Rieske, mitochondrial | *Caenorhabditis elegans* | Mouse (monoclonal) | 1/1,000 | Thermo Fisher Scientific |
| RISP     | Rieske iron-sulfur protein | *Arabidopsis thaliana* | Rabbit (polyclonal) | 1/5,000 | Gift of Prof. Ian Small, UWA |
| Accession     | Description                        | Source                   | Mitochondrial Location                                          |
|--------------|------------------------------------|--------------------------|---------------------------------------------------------------|
| sp|P40939|ECHA_HUMAN Trifunctional enzyme subunit alpha, mitochondrial| OS=Homo sapiens |GN=HADHA PE=1 | SV=2 | 38 | 82.999 | 7.82E+08 | 5.12E+08 | 2.39E+09 |
| sp|Q8NI60|COQ8A_HUMAN Atypical kinase COQ8A, mitochondrial| OS=Homo sapiens |GN=COQ8A PE=1 | SV=1; | sp|Q60936|COQ8A_MOUSE Atypical kinase COQ8A, mitochondrial| OS=Homo sapiens |GN=COQ8A PE=1 | SV=2 |
| sp|Q96MF6|CQ10A_HUMAN Coenzyme Q-binding protein COQ10 homolog A, mitochondrial| OS=Homo sapiens |GN=COQ10A PE=2 | SV=2 | 4 | 27.686 | 2811900 | 625990 | 1910800 |
| sp|P30044|PRDX5_HUMAN Peroxiredoxin-5, mitochondrial| OS=Homo sapiens |GN=PRDX5 PE=1 | SV=4; | sp|P99029|PRDX5_MOUSE Peroxiredoxin-5, mitochondrial| OS=Mus musculus |GN=Prdx5 PE=1 | SV=2 |
| sp|Q8IYU8|MICU2_HUMAN Calcium uptake protein 2, mitochondrial| OS=Homo sapiens |GN=MICU2 PE=1 | SV=2 | 2 | 2 | 49.666 | 2110300 | 1060400 | 1730000 |
| sp|Q9BV79|MECR_HUMAN Enoyl-[acyl-carrier-protein] reductase, mitochondrial| OS=Homo sapiens |GN=MECR PE=1 | SV=2 | 2 | 0 | 40.461 | 1806200 | 0 | 5680900 |
| sp|Q7Z3D6|GLUCM_HUMAN D-glutamate cyclase, mitochondrial| OS=Homo sapiens |GN=DGLUCY PE=1 | SV=2 | 1 | 1 | 66.436 | 0 | 431920 | 385680 |
| sp|Q9NYY8|FAKD2_HUMAN FAST kinase domain-containing protein 2, mitochondrial| OS=Homo sapiens |GN=FASTKD2 PE=1 | SV=1 | 1 | 0 | 81.462 | 126130 | 0 | 0 |
| sp|Q9UJZ1|STML2_HUMAN Stomatin-like protein 2, mitochondrial| OS=Homo sapiens |GN=STOML2 PE=1 | SV=1 | 1 | 0 | 38.534 | 345700 | 0 | 0 |
| sp|Q9Y3E5|PTH2_HUMAN Peptidyl-tRNA hydrolase 2, mitochondrial| OS=Homo sapiens |GN=PTRH2 PE=1 | SV=1 | 1 | 0 | 19.193 | 1142800 | 0 | 1483400 |
| sp|Q96E52|OMA1_HUMAN Metalloendopeptidase OMA1, mitochondrial| OS=Homo sapiens |GN=OMA1 PE=1 | SV=1 | 0 | 0 | 60.12 | 0 | 0 | 154650 |
| sp|Q96PE7|MCEE_HUMAN Methylmalonyl-CoA epimerase, mitochondrial| OS=Homo sapiens |GN=MCEE PE=1 | SV=1 | 0 | 0 | 18.749 | 0 | 0 | 5553000 |
| sp|P12235|ADT1_HUMAN ADP/ATP translocase 1| OS=Homo sapiens |GN=SLC25A4 PE=1 | SV=4; | sp|P48962|ADT1_MOUSE ADP/ATP translocase 1 | OS=Mus musculus |GN=Slc25a5 PE=1 | SV=3; |
| sp|P45880|VDAC2_HUMAN Voltage-dependent anion-selective channel protein 2| OS=Homo sapiens |GN=VDAC2 PE=1 | SV=2 | 3 | 2 | 31.566 | 30975000 | 9429100 | 2.06E+08 |
| sp|P51881|ADT2_MOUSE ADP/ATP translocase 2| OS=Mus musculus |GN=Slc25a5 PE=1 | SV=3; | sp|P05141|ADT2_HUMAN ADP/ATP translocase 2 | OS=Homo sapiens |GN=SLC25A5 PE=1 | SV=4; |
| sp|Q92506|DHB8_HUMAN Estradiol 17-beta-dehydrogenase 8| OS=Homo sapiens |GN=HSD17B8 PE=1 | SV=2 | 2 | 0 | 26.973 | 1431400 | 0 | 7073400 |
| sp|Q9BUR5|MIC26_HUMAN MICOS complex subunit MIC26| OS=Homo sapiens |GN=APOO PE=1 | SV=1 | 2 | 0 | 22.284 | 1749400 | 0 | 912210 |
| sp|Q5RI15|COX20_HUMAN Cytochrome c oxidase protein 20 homolog| OS=Homo sapiens |GN=COX20 PE=1 | SV=2 | 1 | 0 | 13.291 | 2932700 | 0 | 0 |

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reviewed ... In adult muscle, NMJ localization depends upon ANK2 presence, but not in newborn animals. {ECO:0000250|UniProtKB:P11531}.

reviewed ... asm. Nucleus. Note=After cleavage, the intracellular domain (LRPICD) is detected both in the cytoplasm and in the nucleus.

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reviewed SUBCELLULAR LOCATION: Cytoplasm {ECO:0000269|PubMed:19464326}. Nucleus {ECO:0000269|PubMed:19464326}. Secreted {ECO:0000269|PubMed:22427880}. Note=Translocates to nuclear foci during heat shock. {ECO:0000269|PubMed:19464326}.

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reviewed... c reticulum's terminal cisternae luminal spaces of cardiac and slow skeletal muscle cells. {ECO:0000250|UniProtKB:O09161}.

reviewed SUBCELLULAR LOCATION: Cell membrane, sarcolemma; Single-pass type II membrane protein. Cytoplasm, cytoskeleton.

reviewed SUBCELLULAR LOCATION: Cell inner membrane {ECO:0000255|HAMAP‐Rule:MF_01347}; Peripheral membrane protein {ECO:0000255|HAMAP‐Rule:MF_01347}.

reviewed... rane {ECO:0000269|PubMed:11276205}. Note=Identified by mass spectrometry in melanosome fractions from stage I to stage IV.

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reviewed SUBCELLULAR LOCATION: Peroxisome {ECO:0000250|UniProtKB:Q9D5T0}. Cell junction, synapse, postsynaptic cell membrane {ECO:0000250|UniProtKB:Q9D5T0}.

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reviewed SUBCELLULAR LOCATION: Cytoplasm, cytoskeleton {ECO:0000269|PubMed:9230079}. Cell projection {ECO:0000269|PubMed:9230079}.

reviewed SUBCELLULAR LOCATION: Cell membrane {ECO:0000305}; Multi‐pass membrane protein {ECO:0000305}.

reviewed

reviewed... ardiomyocytes. Centrosome. Localizes to the centrosomes in a microtubule‐dependent manner (By similarity). {ECO:0000250}.

reviewed SUBCELLULAR LOCATION: Cytoplasm, cytosol {ECO:0000269|PubMed:20801876}. Cytoplasm, cytoskeleton {ECO:0000269|PubMed:20801876}. Membrane {ECO:0000269|PubMed:20801876}. Note=Tightly but non‐covalently associated with membranes.

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reviewed... 206990}. Note=Secretion is stimulated by TSH/thyroid‐stimulating hormone, INS/insulin and SST/somatostatin. {ECO:0000250}.
| Accession | Description                  | Species          | Enzyme | Protein | Source   | Mass     | Charge |
|-----------|------------------------------|------------------|--------|---------|----------|----------|--------|
| A0A0D3DZN2 | Malic enzyme                 | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 67.047   | 2.81E+08 | 1.33E+08 |
| A0A0D3BD05 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 61.344   | 2.78E+08 | 1.16E+08 |
| A0A0D3AG28 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 59.04    | 5.87E+09 | 9.77E+08 |
| A0A0D3AW54 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3E0G3 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3BDT3 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 57.19    | 10036000 | 2861900  |
| A0A0D3A4L7 | Elongation factor Tu         | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3DF63 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 79.4     | 7436000  | 5486100  |
| A0A0D3CBG7 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 11.137   | 2255900  | 788730   |
| A0A0D3CBA0 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 35.712   | 581770   | 0        |
| A0A0D3AJ74 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3EFB2 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 108.18   | 776290   | 0        |
| A0A0D3EFM2 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 39.241   | 9465200  | 25927000 |
| A0A0D3CBL6 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3DQG2 | Uncharacterized protein (Fragment) | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 38.131   | 3439800  | 29303000 |
| A0A0D3BZU0 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 49.631   | 306070   | 255260   |
| A0A0D3BME5 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 111.89   | 452170   | 527570   |
| A0A0D3BJZ3 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 46.177   | 575200   | 2940400  |
| A0A0D3BJI7 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 74.121   | 4146600  | 1253500  |
| A0A0D3A154 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 72.947   | 9938200  | 9677600  |
| A0A0D3DR38 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 58.628   | 4438300  | 2677600  |
| A0A0D3BYK1 | Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 68.965   | 4177300  | 5176300  |
| A0A0D3A1B3 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          | 87.655   | 46551    | 29759    |
| A0A0D3E0G6 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3DB82 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 30.33    | 466340   | 0        |
| A0A0D3C124 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 83.505   | 1190000  | 1.7E+08  |
| A0A0D3B6Y5 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 27.908   | 2706400  | 1436800  |
| A0A0D3C6J2 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3B6Q1 | Citrate synthase             | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3C679 | Citrate synthase             | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3ACD4 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3BC46 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3BJJ9 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 30.902   | 579300   | 1093500  |
| A0A0D3BHV7 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          | 36.93    | 562580   | 1083200  |
| A0A0D3E0I0 | Chlorophyll a‐b binding protein, chloroplastic | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3CPS0 | Phosphotransferase           | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3CG17 | Phosphotransferase           | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3B0D5 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3AIB0 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3CU21 | Ferritin                     | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3BTY2 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=3   | SV=1    |          |          |         |         |
| A0A0D3CV59 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3AKY4 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D2ZW19 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3B2I1 | Sulfurtransferase            | Brassica oleracea | GN=B77C13.7 | PE=4 | SV=1 |          | 41.534 | 2108500 | 1515200 |
| A0A0D3CAS2 | Sulfurtransferase            | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D3AKY4 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |
| A0A0D2ZW19 | Uncharacterized protein      | Brassica oleracea var. oleracea | PE=4   | SV=1    |          |          |         |         |

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Effects of astaxanthin-rich *Haematococcus pluvialis* extract on cognitive function: a randomised, double-blind, placebo-controlled study

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In this study we tried to confirm the effect of an astaxanthin-rich *Haematococcus pluvialis* extract on cognitive function in 96 subjects by a randomised double-blind placebo-controlled study. Healthy middle-aged and elderly subjects who complained of age-related forgetfulness were recruited. Ninety-six subjects were selected from the initial screen, and ingested a capsule containing astaxanthin-rich *Haematococcus pluvialis* extract, or a placebo capsule for 12 weeks. Somatometry, haematology, urine screens, and CogHealth and Groton Maze Learning Test were performed before and after every 4 weeks of administration. Changes in cognitive performance and the safety of astaxanthin-rich *Haematococcus pluvialis* extract administration were evaluated. CogHealth battery scores improved in the high-dosage group (12 mg astaxanthin/day) after 12 weeks. Groton Maze Learning Test scores improved earlier in the low-dosage (6 mg astaxanthin/day) and high-dosage groups than in the placebo group. The sample size, however, was small to show a significant difference in cognitive function between the astaxanthin-rich *Haematococcus pluvialis* extract and placebo groups. No adverse effect on the subjects was observed throughout this study. In conclusion, the results suggested that astaxanthin-rich *Haematococcus pluvialis* extract improves cognitive function in the healthy aged individuals.

Key Words: Astaxanthin, *Haematococcus pluvialis*, cognitive function, aging, clinical efficacy

It is widely accepted that cognitive function decreases with aging. Moreover, dementia, Alzheimer disease, etc., are diseases in which cognitive function is drastically reduced. Astaxanthin (Ax) is a carotenoid found in marine organisms such as shrimp, crab, krill, salmon, and microalgae. It has strong anti-oxidant properties, as it consumes free radicals such as singlet oxygen to form stable triplet oxygen. In recent years, the effect of Ax against aging and against illnesses related to oxidisation stress. Ax exhibits immunomodulation properties and alleviates eye fatigue and prevents *Helicobacter pylori* infection. Ax exhibits anti-apoptotic properties and reduces caspase 3 and 9 activation in neuroblastoma cells exposed to oxidative stress. Ax also inhibits oxidative stress-induced apoptosis in nerve cells. Memory and learning ability, as assessed in the T-maze test, is improved in immature mice by treatment with Ax. Moreover, when a normal aging mouse was medicated with Ax, memory improvement was observed. In Morris water maze examinations in Fischer rats, Ax treatment improved the memory of both immature (4 months) and aged rats (18 months). The effect was more remarkable in the old rats than in the young ones, suggesting that Ax improves cognition and/or prevents age-related cognitive damage.

We performed a preliminary clinical trial of 12 weeks of astaxanthin-rich *Haematococcus pluvialis* extract (Ax-Hp) treatment in 10 healthy men between 50 to 69 years of age, who complaints of age-related forgetfulness to assess the effects of the supplements on cognition. We reported changes in the P300 brain waves, related to cognitive function, and improvements in CogHealth scores. This randomised double-blind placebo-controlled study was performed to validate the effects of Ax-Hp on cognitive function. In the preliminary trial, a 12-mg/day dose of Ax elicited an effect. In this trial, we employed a 12-mg/day dose and a 6-mg/day dose to look for the presence of a dose response.

Materials and Methods

Study subjects. Healthy men and women between 45 to 64 years of age, who complaints of age-related forgetfulness were recruited; 138 subjects participated after providing signed informed consent. The subjects’ height, body weight, body mass index (BMI), blood pressure, and pulse rate were measured, along with performing haematological and urinary analyses, the Hachinski Ischaemic Scale, and tests of cognitive function (Hasegawa Dementia Scale-Revised [HDS-R], CogHealth, and Groton Maze Learning Test [GMLT]).

Subjects were excluded if they exhibited signs of dementia (HDS-R score, ≥20 points) or cerebrovascular dementia (Hachinski Ischaemic Score, ≥7), habitually consumed Ax supplements,

**Key Words:** Astaxanthin, *Haematococcus pluvialis*, cognitive function, aging, clinical efficacy

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used games and books designed to improve cognitive function, or were judged unfit for participation due to the results of their laboratory analyses. Eventually, 96 subjects (46 men and 50 women, 55.7 ± 3.7 years of age) qualified for participation.

**Study design.** This randomised double-blind placebo-controlled study consisted of a high-dosage group (12 mg/day of Ax), low-dosage group (6 mg/day of Ax), and a placebo group. The subjects were randomly allocated into 3 groups (n = 32 per group). After confirming that the average age and BMI of each group were equivalent, the study coordinator named the 3 groups as the high-dosage group, low-dosage group, and placebo group, and created the allocation table. The coordinator also labelled the high-dosage, low-dosage, and placebo supplements with the subject IDs, according to the allocation table. The coordinator sealed and stored the allocation table until the study ended. The demographic characteristics of each group are shown in Table 1.

To minimise the learning effect, the CogHealth battery and GMLT were repeated 2 weeks after the screening tests; these scores were set as the baseline. Assessments of subjective symptoms, somatometry, haematological and urinary tests, and cognitive function tests (CogHealth and GMLT) were performed after 4, 8, and 12 weeks of Ax-Hp administration. The subjects maintained a diary to record their daily Ax-Hp supplement doses and the subjective symptoms during the 12-week study period (Fig. 1).

**Supplements.** Puresta® (YAMAHA Motor Co., Ltd.) was used for Ax-rich Haematococcus pluvialis oil. The raw materials of the supplements were olive oil (K. Kobayashi & Co., Ltd.), gelatine (porcine in origin), Ax-Hp (6 or 12 mg of Ax dialcohol), glycerine, vitamin E, and an emulsifier. The placebo capsule had corn oil (J-OIL MILLS, Inc.) as a substitute for Ax-Hp. The subjects ingested the supplement after breakfast everyday for 12 weeks. When they could not take the supplement after breakfast, they were asked to take it after lunch or supper.

**Ethics.** The study protocol and informed consent documents were reviewed and approved by the ethics committee of Anti-Aging Science, Inc. All study subjects provided written informed consent prior to participation. The protocols were carried out under the provisions of the Declaration of Helsinki.

**Cognitive function tests.**

- **CogHealth battery.** The CogHealth battery measures response time and accuracy with 5 card games played on a personal computer. The card games include tests of ‘simple reaction’, ‘choice reaction’, ‘working memory’, ‘delayed recall’, and ‘divided atten-

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**Table 1. Characteristics of study subjects**

| Characteristic      | Placebo group | Ax-Hp low-dosage group | Ax-Hp high-dosage group |
|---------------------|---------------|------------------------|-------------------------|
| No. of subjects     | 32            | 32                     | 32                      |
| No. of men/women    | 15/17         | 16/16                  | 15/17                   |
| Age (years)         | 51.6 ± 5.3    | 51.1 ± 5.9             | 51.5 ± 5.7              |
| Height (cm)         | 161.6 ± 7.0   | 161.8 ± 8.4            | 161.4 ± 8.9             |
| Body weight (kg)    | 61.1 ± 10.1   | 61.0 ± 10.8            | 62.6 ± 11.6             |
| BMI                 | 23.3 ± 2.8    | 23.2 ± 2.9             | 23.9 ± 3.3              |

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Fig. 1. Flow diagram of the experimental design and procedure.
The ‘simple reaction’ task requires the subjects to push a button as quickly as possible when the cards are placed face-up on a table. The ‘choice reaction’ task requires subjects to identify whether a card is red or black by pushing a button (labelled YES or NO) when cards are placed face-up on a table. These tasks measure the reaction and control in a frontal lobe function. The ‘working memory’ task requires subjects to identify whether a card is the same or different from the previous card. The ‘delayed recall’ task requires subjects to identify whether overturned cards have appeared previously. These 2 tasks measure immediate memory and episodic memory. The ‘divided attention’ task requires subjects to identify whether a card touches a line while moving up and down at random. This task measures spatial attention.

The response time is measured with a sensitivity of 1/1,000 s. The CogHealth battery is not influenced by culture, language, or level of education, and is not influenced by the learning effect. Moreover, it can detect a slight change in cognitive function and can be used to diagnose mild cognitive impairment. The CogHealth test is based on task switching as an evaluation of high-order cognitive functions (execution function), such as control of action or reconstruction of information processing. Moreover, brain image analysis by fMRI has revealed frontal lobe activity associated with task switching; thus, the CogHealth test is also a frontal lobe function test.

Reaction time in a task-switching test is a measure of the processing time in total cognition and performance processing, and is widely used as an index of cognitive information-processing ability.

Table 2. Comparison of the 3 groups of CogHealth at the time of Ax-Hp administration

| Task               | Time | p value | Group | Interaction |
|--------------------|------|---------|-------|-------------|
| Response time      |      |         |       |             |
| Simple reaction    | 0.376| 0.249   | 0.817 |             |
| Choice reaction    | 0.601| 0.770   | 0.826 |             |
| Working memory     | 0.220| 0.636   | 0.436 |             |
| Delayed recall     | 0.174| 0.552   | 0.423 |             |
| Divided attention  | 0.621| 0.467   | 0.810 |             |
| Accuracy           |      |         |       |             |
| Working memory     | 0.074| 0.892   | 0.178 |             |
| Delayed recall     | 0.343| 0.344   | 0.635 |             |

Table 3. Mean response times and accuracies (±SD) on CogHealth tasks at baseline, and after 4, 8, and 12 weeks of Ax treatment

| Group/Task       | Baseline | 4 weeks | 8 weeks | 12 weeks |
|------------------|----------|---------|---------|----------|
|                  | Mean ± SD| Mean ± SD| Mean ± SD| Mean ± SD|
| Simple reaction  | 288.7 ± 59.1| 271.5 ± 38.2| 0.789| 267.8 ± 40.6| 0.345| 265.0 ± 36.9| 0.123|
| Choice reaction  | 467.5 ± 70.9| 446.4 ± 53.1| 0.506| 453.1 ± 56.5| 1.000| 440.9 ± 52.8| 0.124|
| Working memory   | 686.0 ± 148.9| 647.7 ± 100.4| 0.194| 670.6 ± 151.5| 1.000| 644.6 ± 124.7| 0.071∗|
| Delayed recall   | 909.7 ± 218.1| 875.3 ± 217.8| 1.000| 887.8 ± 243.1| 1.000| 903.8 ± 270.1| 1.000|
| Divided attention| 413.0 ± 103.3| 390.0 ± 75.2| 0.597| 379.6 ± 82.0| 0.153| 388.7 ± 73.9| 0.397|
| Working memory   | 95.1 ± 5.3 | 96.2 ± 3.8 | 1.000 | 95.2 ± 5.5 | 1.000 | 94.9 ± 8.3 | 1.000 |
| Delayed recall   | 66.9 ± 10.3 | 68.8 ± 8.6 | 1.000 | 71.1 ± 10.4 | 0.355 | 69.2 ± 9.4 | 1.000 |

Table 4. Comparison of the 3 groups of CogHealth at the time of Ax-Hp administration

| Task               | Group | Interaction |
|--------------------|-------|-------------|
| Simple reaction    |       |             |
| Choice reaction    |       |             |
| Working memory     |       |             |
| Delayed recall     |       |             |
| Divided attention  |       |             |
| Working memory     |       |             |
| Delayed recall     |       |             |

Dropouts were excluded from the data analysis. Data were analyzed by one-way repeated measure ANOVA adjusted for age and sex.
skills. Prior to performing each task, the subjects received a full explanation of the task and were permitted to practice.

GMLT. The ‘maze’ of the GMLT was specified at random on a personal computer in a 10 × 10 layout requiring 28 steps to move from the upper left to the lower right goal. The subjects worked through the same hidden maze 5 times, and then performed 5 CogHealth tasks. The same maze was performed once again at the end of testing, to provide a measure of spatial working memory. The GMLT indicates a learning effect, while the CogHealth is not affected by learning.

In order to exclude the influence of computer skills, the subjects performed the test on a touch screen. The subjects received instructions for all tasks of CogHealth battery and GMLT, were asked to ‘Please push a button quickly and correctly’, and were permitted to practice. Instructions for all tasks of CogHealth battery and GMLT, were performed the test on a touch screen. The subjects received explanation of the task and were permitted to practice. The GMLT indicates a learning effect, while the CogHealth is not affected by learning.

Analysis of the results of CogHealth battery included mean response time (ms) for every task, mean accuracy (%) of ‘working memory’ and ‘delayed recall’, and those of the results of the GMLT included mean total duration (s) and total errors after performing a maze 6 times.

Somatometry. Somatometry data included height, body weight, BMI, blood pressure (systolic/diastolic), and pulse rate.

Haematological and urinary tests. Haematological parameters, including white blood cell count, red blood cell count, haemoglobin level, haematocrit, platelet count, MCV, MCH, and MCHC; biochemical parameters, including levels of total protein, albumin, total bilirubin, triglycerides, total cholesterol, HDL cholesterol, LDL cholesterol, BUN, creatinine, AST, ALT, γ-GTP, LDH, ALP, uric acid, serum electrolytes (Na, K, and Cl), and fasting blood sugar and A/G ratio; and qualitative urinalysis parameters, including protein, glucose, occult blood, pH, and urobilinogen, were determined.

Statistical analysis. All cognitive parameters of CogHealth and GMLT were compared between groups with 2-way factorial ANOVA adjusted for age and sex. One-way repeated measure ANOVA, adjusted for age and sex, was used to compare scores at baseline and at after 4, 8, and 12 weeks; multiple comparisons were performed using Bonferroni correction.

All statistical analysis was performed using SPSS 16.0J for Windows (SPSS Japan Inc., Tokyo, Japan).

Results

The effectiveness and safety of Ax-Hp were evaluated in 89 subjects who participated in all tests (high-dosage group, 29; low-dosage group, 29; and placebo, 31). The subjects ingested 80% or more of the provided Ax-Hp supplements.

Improvemen of CogHealth score. Table 2 shows the comparison of 3 groups at the time of Ax-Hp administration, and Table 3 shows the mean response time of all 5 CogHealth tasks and the mean accuracy of 2 tasks—‘working memory’ and ‘delayed recall’—at baseline and at 4, 8, and 12 weeks of Ax-Hp administration. The 3 groups did not significantly differ. However, the changes in response time were as follows: in the high-dosage group, improvement trends were observed for ‘choice reaction’ (451.1 ± 56.7 vs 480.1 ± 77.5), ‘delayed recall’ (818.3 ± 195.9 vs 880.1 ± 189.4), and ‘divided attention’ (385.3 ± 72.5 vs 419.4 ± 79.6), each p value <0.1 at 12 weeks, and ‘working memory’ was significantly improved (609.2 ± 123.5 vs 655.9 ± 136.5; p<0.05) at 12 weeks; improvement trends were observed for ‘working memory’ (644.6 ± 124.7 vs 686.0 ± 148.9; p=0.1) at 12 weeks in the placebo group and ‘delayed recall’ (844.4 ± 103.8 vs 912.2 ± 145.6; p=0.1) at 8 weeks in the low-dosage group. ‘Delayed recall’, a measure of accuracy, significantly improved in the high-dosage group at 12 weeks (72.9 ± 7.5 vs 67.3 ± 11.8; p<0.05). The results of the CogHealth test suggest that 12 weeks of high-dose Ax-Hp administration improved cognitive function.

Improvement of GMLT score. Table 4 shows the comparison of 3 groups at the time of Ax-Hp administration, and Table 5 shows the total durations and errors of the 6 trials of GMLT at baseline and at 4, 8, and 12 weeks of Ax-Hp administration. There was no significant difference in the 3 groups. In each group, the total duration was significantly shortened at 8 weeks, but there was no difference between the groups.

However, the total errors in each group were as follows: the low-dosage group showed significant improvement after 4 weeks (63.3 ± 24.5 at Week 4, 61.3 ± 21.6 at Week 8, and 57.3 ± 20.7 at Week 12, each p<0.01 vs baseline; 79.9 ± 31.0); the high-dosage group also showed significant improvement after 4 weeks (68.9 ± 33.9; p<0.05 at Week 4, and 60.8 ± 24.5; p<0.01 at Week 8 and 60.6 ± 25.1; p<0.01 at Week 12, each p values vs baseline; 83.0 ± 36.9), whereas the placebo group showed significant improvement at 12 weeks (60.7 ± 25.6 vs 74.3 ± 22.9; p<0.01).

On the basis of these results, we conclude that short-term spatial working memory was improved by ingestion of 6 mg of Ax.

Safety evaluation of Ax-Hp. Somatometry, haematological and urinary tests, and an oral consultation after 12 weeks of Ax-Hp administration.

Table 4. Comparison of the 3 groups of GMLT at the time of Ax-Hp administration

| Task       | Time | Group | Interaction |
|------------|------|-------|-------------|
| Total duration | 0.185 | 0.850 | 0.870       |
| Total errors | 0.724 | 0.905 | 0.278       |

Table 5. Mean total duration and total errors (±SD) on GMLT tasks at baseline, and after 4, 8, and 12 weeks of Ax treatment

| Group/Task | Baseline | 4 weeks | p (vs baseline) | 8 weeks | 12 weeks | p (vs baseline) |
|------------|----------|---------|----------------|---------|----------|----------------|
| Placebo group (n=31) | | | | | | |
| Total duration (s) | 158.8 ± 36.0 | 148.7 ± 35.6 | 0.193 | 143.0 ± 34.9 | 0.017* | 134.4 ± 33.2 | <0.001** |
| Total errors | 74.3 ± 22.9 | 67.8 ± 23.3 | 0.925 | 65.1 ± 23.0 | 0.258 | 60.7 ± 25.6 | 0.003** |
| Ax low-dosage group (n=29) | | | | | | |
| Total duration (s) | 157.8 ± 25.9 | 147.3 ± 21.5 | 0.322 | 137.2 ± 23.8 | 0.002** | 127.8 ± 16.2 | <0.001** |
| Total errors | 79.9 ± 31.0 | 63.3 ± 24.5 | 0.005* | 61.3 ± 21.6 | 0.001** | 57.3 ± 20.7 | <0.001** |
| Ax high-dosage group (n=29) | | | | | | |
| Total duration (s) | 162.0 ± 44.8 | 152.2 ± 43.0 | 0.517 | 139.5 ± 34.3 | <0.001** | 135.4 ± 33.2 | <0.001** |
| Total errors | 83.0 ± 36.9 | 68.9 ± 33.9 | 0.024* | 60.8 ± 24.5 | <0.001** | 60.6 ± 25.1 | <0.001** |

Dropouts were excluded from the data analyses. Data were analyzed by one-way repeated measure ANOVA, adjusted for age and sex. Multiple comparisons of 4, 8, and 12 weeks with baseline were performed using Bonferroni correction.
administration revealed no confirmed adverse effects, indicating that Ax-Hp supplementation is safe.

Discussion

There have been many reports of research on cognitive functional improvement in the fields of medicine, psychology, and exercise physiology. The aging population and the medical issues that accompany aging have raised concerns about cognitive function improvement. And it is hoped that prevention may be achieved via specific dietary changes; there have been promising reports on the effectiveness of docosahexaenoic acid, (25) arachidonic acid, (26) Ginkgo biloba, (20) Pinus radiata bark extract, (30) and acetic acid bacteria (31) in retarding cognitive function loss.

Ax is a strong anti-oxidant. Nakagawa et al. (32) reported the anti-oxidant effect of Ax-Hp on phospholipid peroxidation in human erythrocytes. Iwabayashi et al. (33) reported that Ax-Hp increases the biological anti-oxidants potential (BAP) in human. It is clear that the anti-oxidant activity of Ax-Hp is effective in human and animal studies. This study lets expect that Ax-Hp reduces oxidation in the brain, leading to improved scores in tests of cognitive function.

Flavonoids are strong natural anti-oxidants like Ax, and they were thought to improve age-related cognitive decline. Youdim et al. (34) reported on the neuroprotective effects of dietary flavonoids in vivo. Pipingas et al. (35) reported that flavonoid-rich P. radiata bark extract improved cognitive function in tests of immediate recognition and spatial working memory. It is thought that the brain and nervous oxidation are improved by the anti-oxidant activity of flavonoids, as is the case with Ax-Hp.

We performed a preliminary clinical trial of Ax-Hp and cognitive function improvement in 10 healthy men between 50 to 69 years of age, who complained of age-related forgetfulness. We reported that the response time of 5 CogHealth tasks was significantly improved ($p<0.05$) and that the amplitude of P300 brain waves, which are related to cognitive function, tended to increase ($p<0.1$) after 12 weeks of Ax-Hp treatment.

This randomised double-blind placebo-controlled study was performed to validate the beneficial effects of Ax-Hp on cognitive function in human subjects. Based on the results of the preliminary study, the high dosage was set at 12 mg/day, and 6 mg/day was set as the low dosage in order to confirm the presence of a dose response. The administration period was set to 12 weeks as in the preliminary study. Brain function was assessed with the CogHealth test and GMLT, both of which can perform objective measures in a large number of subjects.

We failed to show a significant difference between groups in the CogHealth test. No differences were observed at any of the assessments (4, 8, or 12 weeks). In addition, there were no differences between the high-dosage, low-dosage, and placebo groups in the reaction time and accuracy of the task-switching test. However, we observed significant improvements in the high-dose group in the response time for 1 task and in the accuracy of 1 task, and improvement trends was observed in 3 tasks. We conclude that these significant differences and trends are indicative of the Ax-induced improvement in cognitive function.

GMLT also revealed no significant differences between groups. The total duration did not change between groups or over time. However, total error improved significantly by 4 weeks in the low-dosage and high-dosage groups in contrast to the placebo group, which showed significant improvement only at 12 weeks. These results also suggest the Ax-induced improvement in cognitive function.

Although improvements in the CogHealth test were observed only with a dose of 12 mg/day, GMLT scores were improved at 6 mg/day. This difference may be because the methods of measuring cognitive performance differ. The GMLT includes a test of spatial working memory that is highly sensitive, can be used to measure age-related reductions in cognitive function, and can detect the effect of a supplement or remedy. Further verification is required.

Although the tests employed by Pipingas et al. (36) differ from the tests used in this study, immediate recognition is evaluated by the CogHealth test and spatial working memory is evaluated by the GMLT. The combination of these tasks may be useful to verify other supplements or remedies.

The CogHealth test revealed improvements in cognitive function with 12 mg/day Ax-Hp for 12 weeks. This supports the results of our preliminary clinical study. In particular, the improvement in response time, which is a measure of short-term memory, and in the accuracy of the ‘delayed recall’ task was remarkable. Moreover, total errors in the GMLT, which is also associated with memory, showed significant improvement with 6 and 12 mg/day Ax-Hp.

However, significant differences between groups were not observed, possibly due to the small sample size. Moreover, the average age may have been too young to observe age-related cognitive decline. We plan to investigate this issue further.

We observed no adverse effects to express any concerns regarding the safety of Ax-Hp.

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Conflicts of Interests

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Effects of astaxanthin supplementation in healthy and obese dogs

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Background: Since astaxanthin (ASX) has potent anti-oxidative effects with inhibitory action of lipid peroxidation and singlet oxygen quenching activity, it is widely used as a functional food for keeping good health in human. Obesity is a risk factor for various metabolic disorders. It is characterized by low-grade chronic inflammation based on oxidative stress by excessively produced ROS. From the point of preventive medicine, natural compounds have been proposed as potential therapeutic agents in the prevention of metabolic disorder in companion animals. The purpose of this study is to evaluate the effects of ASX supplementation in healthy and obese dogs.

Materials and methods: Ten healthy beagle dogs and 5 clinically obese dogs were used in this study. The healthy beagle dogs were randomly divided into 2 groups as follows: control and test groups. The test group dogs received ASX supplementation mixed with the food for 6 weeks. Five clinically obese dogs received ASX supplementation for 8 weeks. Metabolites, hormones and enzymes were measured before and after ASX supplementation.

Results: In the healthy dog groups, after 6 weeks, plasma triglyceride (TG) and malondialdehyde concentrations and lactate dehydrogenase (LDH) values significantly decreased in the test group. There was no significant difference in the control group. In clinically obese dogs, plasma TG concentration decreased after 8 weeks of ASX supplementation. Plasma alanine aminotransferase and LDH values clearly decreased in all 5 dogs and 4 dogs out of 5 dogs, respectively.

Conclusion: ASX supplementation (0.3 mg/kg body weight/day) for 6 weeks in healthy dogs and 8 weeks in obese dogs induced the elevation of antioxidant function and of liver function by ameliorating lipid metabolism.

Keywords: astaxanthin, obese dogs, lipid metabolism, anti-oxidative activity, liver function

Introduction

The incidence of obesity and its associated diseases has been increased in dogs and cats as well as in human. 1, 2 Since obesity causes physical inactivity and oxidative stress related diseases that are induced by obesity-based metabolic syndrome, body weight (BW) reduction is required for obese animals. However, the satisfactory result of weight reduction is limited in the animals with pathological obesity induced by accumulated visceral fat, causing slight systemic inflammation. 3 Fat accumulation and oxidative stress impair the function of mitochondria via morphological alteration, increased membrane peroxidation, decreased ATP level, increased ROS production, defective mitochondrial β-oxidation and increased mitochondrial permeabilization. 4 Increase in circulating nonesterified fatty acids (NEFAs) due to excessive accumulated
visceral fat is confirmed in obese animals. Fat accumulation triggers free radical production and insults additional inflammation. Excess amount of ROS is also produced via accelerated β-oxidation of fatty acids. Such overproduced ROS is attributed to one of the pathogens for obesity and its associated diseases. Consequently, some antioxidants appear to be effective to ameliorate obesity conditions in animals. Experimental studies of mice administered obesity-inducing diet combined with ASX showed anti-diabetic and anti-obesity effects by improved insulin (INS) sensitivities and liver function. The study showed the suppression of fat tissue weight gain by ASX in a dose-dependent manner.

*Haematococcus pluvialis*, known as an important source of natural astaxanthin (ASX), is a freshwater microalga belonging to the family *Chlamydomonadaceae*. When the alga experiences environmental stress conditions, ASX is created and acts like a force field that protects the nuclear DNA and lipids against UV-induced oxidation. ASX (3,3′-dihydroxy-β,β′-carotene-4,4′-dione) is a nontoxic and organic fat-soluble xanthophyll carotenoid. In comparison to other phytochemicals, ASX has previously been reported to possess a significantly greater antioxidant function, with its antioxidant activities quantified as 10-fold greater than other carotenoids, such as β-carotene, and 100-fold greater than α-tocopherol (vitamin E). ASX accumulates in the liver, especially in the microsomal and mitochondrial fractions of the liver tissue. This substance has been shown to prevent oxidative damage to the liver, improve metabolic profiles, and reduce hepatic inflammation. From the above, excessive fat accumulation and oxidative stress and liver function are closely related.

In this study, we measured plasma metabolites and hormone concentrations and enzyme activities involved in energy metabolism in healthy and obese dogs with ASX supplementation for several weeks. The purpose of this study was to evaluate the effect of ASX supplementation in obese and healthy dogs.

### Materials and methods

#### Animals

Ten healthy beagle dogs and 5 clinically obese dogs were used in this study. Their body condition score (BCS) was evaluated by the 5-point scale system (1, very thin; 2, underweight; 3, ideal; 4, overweight and 5, obese). BCS of 10 healthy dogs was 3. The average age of them was 2 years (1–3 years), and the average BW was 10.4 kg (9.7–11.1 kg). They were randomly divided into 2 groups: control group (dog no 1–5) and test group (dog no 6–10). As preparation of the study, they were given commercial diet (Nippon Pet Food Co., Ltd., Tokyo, Japan) for 2 months. The nutrient composition of the food is crude protein 21.7%, crude fat 10.1%, crude ash 6.3%, crude fiber 2.7%, linoleic acid 1.72%, moisture 8.7% and nitrogen-free extract 50.5%. All the dogs were kept under controlled conditions and professionally supervised at Narita Animal Science Laboratory Co., Ltd. (Narita, Japan) prior to and during the study period. Ethical approval for this study was obtained from Narita Animal Science Laboratory Co., Ltd. Research Animal Ethical Committee (17-C042).

Five clinically obese dogs were recruited from local primary veterinary practice as shown in Table 1. The selected 5 dogs met the following 3 conditions: 1) BW must be more than 20% of its ideal weight; 2) symptoms of chronic disease are properly managed and 3) owner is reliable, and good compliance can be received with the complete consent. Case numbers 1, 2 and 3 have been treated for their chronic diseases by certain therapeutic agents for years, and the clinical signs have been well controlled for long time. Case numbers 4 and 5 have taken no medications.

#### ASX supplementation

ASX that is *H. pluvialis* biomass (AstaReal® AW1011; AstaReal Inc., Moses Lake, WA, USA) was used in this study. In the healthy beagle dogs, 5 test group dogs were given 1 dose of

| Case Number | 1 | 2 | 3 | 4 | 5 |
|-------------|---|---|---|---|---|
| Breed       | Miniature Dachshund | Mongrel | Miniature Schnauzer | Kishu dog | Labrador retriever |
| Age (years) | 10 | 14 | 11 | 8 | 3 |
| Sex         | NM | NM | NM | NF | NM |
| Clinical complication | Hypothyroidism | Cushing syndrome | Hypothyroidism, Arthritis | Arthritis | None |
| BW (Kg)     | 10.0 | 11.3 | 15.4 | 15.6 | 25.5 |
| Ideal weight (Kg) | 7 | 11 | 6 | 15 | 35 |
| BCS         | 5 | 5 | 4 | 4 | 5 |

**Abbreviations:** BCS, body condition score; BW, body weight; NF, neutered female; NM, neutered male; W, weeks.
0.3 mg/kg ASX with a meal per day. BW and BCS were measured every week, and the ASX supplement dose was adjusted. For clinical cases, 0.3 mg /kg /day of ASX was given in a single dose or in divided doses with the food on a daily basis.

**Blood sampling**

Fasting blood samples were collected before initiation of the study, after 6 weeks in healthy dogs, and after 8 weeks in clinically obese dogs, respectively. Collected blood was dispensed in a heparinized tube and centrifuged at 400 \( \times g \) for 10 minutes at 4°C to collect plasma. Plasma was stored at −80°C until use.

**Metabolite, hormone and enzyme analyses**

Glucose (GLU), total cholesterol (TC), triglyceride (TG), total protein (TP), blood urea nitrogen (BUN), creatinine (CRE) concentrations and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) activities were measured using an auto-analyzer (JCA-BM2250; JEOL, Tokyo, Japan) and lactate dehydrogenase (LDH) activities were measured using the NEFA-C test kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Plasma malondialdehyde (MDA) concentration was measured using the NWLSS™ Malondialdehyde assay kit (Northwest Life Science Specialties, LLC, Vancouver, Canada). Plasma INS, adiponectin (ADN) and TNFα were measured by the Rat Insulin ELISA kit (AKRIN-010T; Shibayagi Co., Gunma, Japan), mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and TNFα Dog ELISA kit (LS-F1347-1; Life Span Bioscience, Inc, Seattle, WA, USA), respectively.

**Statistical analysis**

All values were calculated using Microsoft Excel. The data were expressed as mean±standard error (SE). Statistical analysis was performed using the 2-tailed, paired \( t \)-test. Statistical significance was designated as \( P<0.05 \), and a high level of significance was designated as \( P<0.01 \).

**Results**

Comparisons of biomarker levels in healthy dogs in the control group and the test group are shown in Table 2. The values of plasma TG and MDA concentrations and LDH significantly decreased in the test group dogs (\( P<0.05 \), \( P<0.01 \)), and there was no significant difference in the control group. After 6 weeks, TGs significantly decreased (\( P<0.05 \)) in the test group, MDA and LDH also significantly decreased in

| Parameter | without ASX (n=5) | 6 weeks | with ASX (n=5) | 6 weeks |
|-----------|-----------------|---------|---------------|---------|
| Body weight (kg) | 10.5 ± 0.2 | 11.3 ± 0.3 | 10.3 ± 0.2 | 11.1 ± 0.3 |
| Body condition score | 3 | 3 | 3 | 3 |
| Glucose (mg/dL) | 87.4 ± 1.8 | 79.8 ± 2.1 | 90.6 ± 2.5 | 87.4 ± 2.3 |
| Triglyceride (mg/dL) | 30.6 ± 1.3 | 38.0 ± 6.6 | 37.2 ± 1.3 | 23.6 ± 4.1 |
| Total cholesterol (mg/dL) | 144.8 ± 20.0 | 131.6 ± 8.9 | 113.8 ± 6.5 | 133.6 ± 9.1 |
| NEFA (mg/L) | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.8 ± 0.0 | 1.0 ± 0.1 |
| Total protein (g/dL) | 7.1 ± 0.1 | 7.0 ± 0.1 | 6.8 ± 0.1 | 6.6 ± 0.1 |
| Blood urea nitrogen (mg/dL) | 13.4 ± 0.7 | 12.0 ± 0.7 | 12.4 ± 0.7 | 10.2 ± 0.6 |
| Creatinine (mg/dL) | 0.7 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.0 | 0.7 ± 0.1 |
| Malondialdehyde (µmol/L) | 0.2 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 |
| Adiponectin (µg/mL) | 18.4 ± 4.0 | 27.5 ± 3.3 | 27.0 ± 6.3 | 30.5 ± 4.3 |
| TNF-α (pg/mL) | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| AST (IU/L) | 32.2 ± 1.8 | 35.2 ± 2.4 | 37.2 ± 1.3 | 33.6 ± 1.5 |
| ALT (IU/L) | 28.5 ± 4.1 | 30.2 ± 5.2 | 31.6 ± 12.2 | 36.4 ± 2.9 |
| ALP (IU/L) | 184.0 ± 46.3 | 166.6 ± 36.0 | 152.4 ± 8.7 | 152.6 ± 8.7 |
| LDH (IU/L) | 83.4 ± 5.7 | 113.6 ± 12.6 | 89.4 ± 6.3 | 57.4 ± 2.4**

*Notes: Data are presented as the mean ± SE. Statistical significance is indicated by asterisks. *Significantly different (\( p<0.05 \)) from the value at 0 week in the test group with ASX (paired \( t \)-test). **Significantly different (\( p<0.01 \)) from the value at 0 week in the test group with ASX (paired \( t \)-test). ***Significantly different (\( p<0.001 \)) from the value at 0 week in the test group with ASX (paired \( t \)-test).*

**Abbreviations:** ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ASX, astaxanthin; LDH, lactate dehydrogenase; NEFA, non-esterified fatty acid; SE, standard error; SE, standard error.
the test group \((P<0.01)\) (Figure 1). Moreover, upon comparing control and test groups in 6 weeks, the values of MDA and LDH significantly decreased \((P<0.01)\) in the test group \((P<0.01)\) (Figure 1). In healthy dogs, with or without ASX supplementation, plasma GLU, TC, NEFA, TP, BUN, CRE, INS, ADN, AST and ALP values showed no significant change before and after 6 weeks of trial (Table 2).

The results of obese dogs are shown in Table 3. TG values clearly decreased after 8 weeks of ASX supplementation, and ALT and LDH values also remarkably decreased in all 5 dogs and in 4 out of the 5 dogs, respectively. Plasma GLU, TC, NEFA, TP, BUN, CRE, INS, ADN, AST and ALP values showed no major change in clinically obese dogs on ASX supplementation after 8 weeks.

Although statistical comparison is impossible, simple comparison of the TG, ALT, MDA and LDH values of the healthy dog groups with individual obese dogs is done (Figure 2). The change before and after ASX supplementation was more pronounced in obese dogs. In each obese dog, ALT values were always higher than those of healthy dogs regardless of ASX supplementation.

BW and BCS showed no changes after ASX supplementation.

**Discussion**

Obesity is characterized by low-grade chronic inflammation.\(^{21}\) This continuous inflammation due to obesity induces severe metabolic disorders such as hypertension, vascular disorders, diabetes mellitus and others.\(^{22}\) Increased circulating NEFAs from accumulated visceral fat cause inflammation and INS resistance by directly activating plasma membrane receptors, such as toll-like receptor 4,\(^{23}\) followed by elevation of inflammatory reaction via NF-κβ.\(^{24,25}\) On the other hand, excessive amount of NEFA enhances overproduction of ROS in the process of β-oxidation of fatty acids in the mitochondria of various tissues. Overproduction of ROS induces oxidative stress. Consequently, systemic inflammatory components were confirmed in obesity.\(^{26,27}\)

Adipose tissue, a population of adipocytes, not only acts as an energy reservoir but also has physiological activities such as angiogenesis and wound healing, and adipocytes produce and secrete adipokines involved in energy metabolism.\(^{28}\) Accumulated visceral fat in obese animals induces high concentrations of plasma NEFA, circulating C-reactive protein (CRP) and MDA.\(^{29,30}\) Excessive amount of visceral fat is suspected as a contributing factor to various metabolic disorders in obese animals. From the above mentioned findings, antioxidant substances are considered to decrease oxidative stress and to ameliorate metabolic disorders caused due to obesity.

Obese animals encountered in veterinary practice are individually unique in their background. In general, dietary supplements and functional foods are often recommended for animals that require the health care intervention from veterinary professionals, such as aging, chronic diseases and...
critical obesity as well. Considering these circumstances, obese dogs participated in this experiment were various age, sex, and species.

In this study, ASX supplementation showed clear antioxidative effects in both healthy and obese dogs. In the healthy dogs, after ASX supplementation for 6 weeks, plasma TG and oxidative stress biomarker MDA concentrations decreased significantly. ASX revealed antioxidant activity in healthy dogs. In the same way, plasma TG, MDA, ALT and LDH values decreased, especially, TG and ALT values remarkably decreased. Antioxidant effects of ASX supplementation in obese dogs are more apparent than those in the healthy dogs. Since those positive effects were observed in all obese dogs with different underlying diseases, it is considered that ASX supplementation could be effective on the antioxidant activity and also improve the hepatic function.

ASX prevents diseases in heart and kidneys\(^{31}\) and liver\(^{20}\) from oxidative stress, in addition ASX can decrease plasma MDA concentrations and improve the pathological signs of animal diabetic nephropathy.\(^{32}\) In this study, ASX supplementation indicated to be effective on elevation of antioxidant function and on amelioration of metabolic functions in liver. Long-term intake of ASX inhibits the elevations in BW and adipose tissue weight caused by a high-fat diet in mice tested for 60 days.\(^{31}\) Chronic ASX administration significantly improve increased body weight, hyperglycemia, hyperinsulinemia and increased plasma levels of TNF\(\alpha\) and IL-6 observed in the study of obese model mice.\(^{33}\) However, ASX supplementation does not influence BW reduction in obese dogs in this study.

In the mice experiments, ASX was given in relatively high dose such as 6, 12 and 30 mg/kg for 60 days. The dose of ASX used in this experiment was set up based on the standard recommended dose to human. Considering the lifespan of the mouse, the duration of 60 days for the mouse corresponds to 1 year or more in the dog. We speculate that longer administration period in obese dogs of more than 2 months could bring the farther positive effect. ASX improved oxidative stress biomarkers by suppressing lipid peroxidation and stimulating the liver function. However, weight reduction effect could not be achieved only by supplementation of ASX to obese dogs.

Moreover, safety of ASX supplementation is advocated by EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in previous report.\(^{34}\) Considering that ASX has wide safety range,\(^{35}\) continuous supplementation of ASX seems to be effective to prevent the prevalence

### Table 3 Changes in biomarkers level of clinically overweight and obese dogs with astaxanthin supplementation

| Case Number | 1 | 2 | 3 | 4 | 5 |
|-------------|---|---|---|---|---|
| **Breed**   | 0W | 8W | 0W | 8W | 0W |
| Miniature Dachshund | 10 | 11.3 | 15.4 | 15.6 | 7.3 | 7.2 | 25.5 | 25.5 | 59.5 | 59.6 |
| Mongrel | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 |
| **Age (years)** | 10 | 14 | 11 | 8 | 3 |
| **Sex** | NM | NM | NM | NM | NM |
| **Clinical complication** | Hypothyroidism | Cushing syndrome | Hypothyroidism, Arthritis | None |
| **Body weight (kg)** | 10 | 4 | 91 | 270 | 450 | 99 | 21 | 18 | 117 | 41 |
| **Body condition score** | 5 | 5 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 |
| **Glucose (mg/dL)** | 87 | 87 | 102 | 87 | 91 | 92 | 74 | 83 | 93 | 104 |
| **Triglyceride (mg/dL)** | 99 | 99 | 178 | 297 | 224 | 230 | 173 | 179 |
| **NEFA (mEq/L)** | 0.54 | 0.45 | 0.51 | 0.58 | 0.63 |
| **Total protein (g/dL)** | 8.2 | 8.1 | 5.8 | 5.7 | 7.6 | 6.8 | 7.4 | 7.3 | 6.6 | 6.6 |
| **Blood urea nitrogen (mg/dL)** | 10 | 16 | 21 | 14 | 11 | 16 | 15 | 14 | 11 |
| **Creatinine (mg/dL)** | 0.6 | 0.6 | 0.4 | 0.4 | 0.4 | 0.3 | 0.7 | 0.7 | 0.9 | 0.8 |
| **Malondialdehyde (μmol/L)** | 6.3 | 2.74 | 3.85 | 1.95 | 10.97 | 3.06 | 1.73 | 1.89 | 3.06 | 2.42 |
| **Insulin (ng/mL)** | 3.6 | 6.7 | 3.5 | 1.0 | 4.7 | 3.2 | 4.3 | 3.8 | 0.5 | 0.7 |
| **Adiponectin (μg/mL)** | 10.8 | 13.9 | 2.0 | 1.5 | NT | 0.14 | 4.8 | 3.4 | 9.7 | 13.3 |
| **AST (IU/L)** | 31 | 39 | 25 | 24 | 211 | 32 | 21 | 18 | 52 | 38 |
| **ALT (IU/L)** | 250 | 270 | 215 | 123 | 74 | 72 | 61 | 135 | 97 |
| **ALP (IU/L)** | 1460 | 352 | 414 | 4215 | 348 | 404 | 2122 | 2130 | 118 | 126 |
| **LDH (IU/L)** | 68 | 173 | 74 | 64 | 7417 | 177 | 73 | 34 | 187 | 76 |

**Abbreviations:** ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ASX, astaxanthin; LDH, lactate dehydrogenase; NEFA, non-esterified fatty acid; NF, neutered female; NM, neutered male; NT, not tested; W, weeks.
of visceral fat-type obesity. Further studies with different doses of ASX supplementation are necessary to clarify the usefulness of ASX as an antioxidant supplement in a large number of dogs with different severities of obesity.

**Conclusion**

ASX supplementation (0.3 mg/kg BW/day) in food for 6 weeks in healthy dogs and for 8 weeks in obese dogs effectively activated antioxidant function and liver function followed by improved lipid metabolism.

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**Disclosure**

Kumi Tominaga is a resercher working in AstaReal Co.Ltd. The authors report no other conflicts of interest in this work.

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**Figure 2** Comparison of TG, MDA, ALT and LDH levels in healthy and obese dogs before and after ASX supplementation.

**Notes:** (A) TG (mg/dL), (B) MDA (μmol/L), (C) LDH (IU/L) and (D) ALT (IU/L). *The value exceeded the display range.

**Abbreviations:** ALT, alanine aminotransferase; ASX, astaxanthin; LDH, lactate dehydrogenase; MDA, malondialdehyde; TG, triglyceride; W, weeks.

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Effect of Astaxanthin supplementation on psychophysiological heart-brain axis dynamics in healthy subjects

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ABSTRACT:

Objective: Marine microalgae is the predominant source of natural astaxanthin (NAX), a red-orange carotenoid with powerful antioxidant and anti-inflammatory properties. Previous studies suggest that NAX supplementation improves antioxidant capacity and reduces oxidative stress, while also enhancing fat utilization, exercise endurance, cardiovascular function, and neurological parameters. The purpose of this study was to assess the effects of NAX on the psychophysiological “heart-brain-axis” while nutrition (astaxanthin) may impact physiology (cardiovascular function) and psychology (mood state) in a coordinated manner.

Methods: Using a double-blind parallel design, 28 healthy subjects (male=14, female=14, age=42) were supplemented for 8 weeks with NAX (12mg/day Haematococcus pluvialis algal extract) or a matching placebo. Before and after supplementation, subjects performed a cardiovascular stress test (VO2max) and completed a validated Profile of Mood States (POMS) survey to assess global mood state (GM) and related subscales: Vigor (V), Tension (T), Depression (D), Anger (A), Fatigue (F), and Confusion (C).

Results: Subjects in the NAX group showed a significant ~10% lower average heart rate at submaximal exercise intensities compared to those in the placebo group (aerobic threshold, AeT; NAX 130±17 v. PL 145±14; and anaerobic threshold, AT; NAX 139±20 v. PL 154±11, p<0.05). Significant improvements were found in the NAX group for both positive mood state parameters: GM (+11%, p<0.05) & V (+5%, NS); and negative mood state parameters: T (~20%, NS), D (~57%, p<0.05), A (~12%, NS), F (~36%, p<0.05), and C (~28%, NS).
Conclusions: NAX supplementation lowered average heart rate at submaximal endurance intensities (suggesting a “physical” heart benefit) and improved mood state parameters (suggesting a “mental” brain benefit). While previous studies have shown NAX supplementation to improve parameters associated with heart health (antioxidant, fat oxidation, endurance) and brain health (neuro-inflammation, cognition, antidepressant/anxiolytic), these results suggest that natural astaxanthin supplementation supports the psychophysiological “heart-brain-axis” with simultaneous improvements in both physical and mental wellness.

Keywords: Antioxidant; Carotenoid; Cardiovascular; Mood State; Mental Wellness

INTRODUCTION

Natural astaxanthin (NAX) is a red-orange carotenoid that provides characteristic color to pink flamingo feathers, pink shrimp shells, and red salmon flesh. The high level of NAX in these tissues is reflective of the animal’s dietary intake of NAX from microalgae, copepods, krill, and other lower food chain organisms [1, 2]. The primary dietary source of astaxanthin for humans is from seafood such as salmon, shrimp, crabs, lobster [3-5]. NAX can exist in 3 stereoisomeric forms [2], two chiral (3S / 3’S and 3R / 3’S), and one meso (3R / 3’S), which are found in nature in variable ratios depending on the diet of the animal (e.g. wild salmon consuming natural algae versus farmed salmon consuming synthetic AX). Synthetic astaxanthin (SAX), derived from petrochemicals and approved as a food coloring for fish aquaculture, is equimolar (3R / 3’S) which results in farmed salmon having high levels of synthetic 3R/3’S, while wild salmon have predominantly 3S/3’S [6].

NAX can be produced commercially from the microalgae Haematococcus pluvialis (H. pluvialis) and is the only source currently approved for human consumption [6] by both the FDA [US Food and Drug Administration] and EFSA [European Food Safety Authority] (both in dried form or extracted with ethanol or supercritical CO2). H. pluvialis as a source of NAX provides the benefits of esterified 3S/3’S astaxanthin stereoisomers (96% of carotenoids) plus other naturally-occurring carotenoids (beta-carotene, lutein, zeaxanthin), which may provide synergistic benefits and improved absorption compared to SAX [7, 8]. In extensive pre-clinical trials, NAX has demonstrated far superior antioxidant capacity in vitro and improved health benefits in animals to SAX [9]. In addition, SAX has not been studied for safety directly in humans [9]. Based on its superiority in bioactivity and extensive safety testing in humans, NAX is the form of Astaxanthin that is used in human nutrition by the vast majority of supplement brands and in functional foods and drinks.

NAX has been studied for its antioxidant, anti-inflammatory, cardioprotective, and neuroprotective activities in humans [10-14]. Rodent studies have further shown NAX to reduce blood pressure and improve blood flow [15, 16], possibly via modulation of cellular stress pathways, including nuclear factor kB (NFkB), nuclear factor E2 related factor 2 (Nrf2), and nitric oxide [17].

In terms of exercise effects, astaxanthin has shown positive results for reducing lactic acid accumulation, increasing fat oxidation/metabolism, and improving endurance performance with most studies demonstrating benefits [18-28]. Many of these effects may be attributed to a
hypothesized mitochondrial-centric mechanism, which could improve energy and redox cellular metabolism (e.g. via Nrf2-ARE pathway activation). Indeed, mitochondrial redox metabolism has been implicated in various neurological disorders including Alzheimer’s, Parkinson’s, and age-related dementia, so it is logical that NAX has been suggested to play a putative prophylactic role in cardiovascular as well as neurologic conditions [29-40].

The heart is recognized to play a vital role not only as a circulatory pump, but as part of a psychophysiological network as a generator and transmitter of system-wide information throughout multiple body systems, including the nervous system [41-46]. Electrical input from the heart can dynamically influence homeostatic, cognitive, perceptual, and emotional processing in the brain, thereby having the potential to affect myriad aspects of mood and behavior [41-44]. Thayer and Lane [41] reviewed the direct and indirect connections between the heart and the brain, including the physiological, behavioral, emotional, and cognitive processes involved in bi-directional control of cortical/cardiac function. Organ crosstalk between the brain/heart has been noted in stress-related cardiomyopathy syndromes and traumatic brain injury [42]. In addition, the use of heart rate variability (HRV) has been recognized for its importance in gauging both the state of the heart (physical stress) as well as the state of the brain (psychological stress). Yoga, as an intervention to restore balanced heart-brain crosstalk through plasticity and stability of the autonomic nervous system, has been used to reduce anxiety levels, atrial fibrillation episodes, blood pressure and neurocardiogenic syncope [43]. Studies have also shown that positive emotional states may improve function of both the cardiovascular and immune systems [45, 46].

MATERIAL AND METHODS
Participants
Thirty healthy, active subjects were recruited and split evenly between males and females. Two subjects, one from each group, withdrew from the study due to issues unrelated to the supplement or trial, resulting in 28 subjects that completed the 8-week supplementation period (age 42±8, range 26-63 years; height 169±10cm; BW 69±6kg). All participants completed informed consent documents approved by an external ethics review board.

Dietary Supplement
Participants were randomly divided in double-blind fashion into two groups to receive either the natural astaxanthin (NAX) supplement (Astazine® Natural Astaxanthin, BGG / AlgaeHealth Sciences) or matching placebo (PL). The NAX supplement provided 12mg/day of natural astaxanthin extracted with ethanol from Haematococcus pluvialis suspended in edible MCT oil (medium chain triglyceride) with d-alpha tocopherol as an antioxidant. Subjects consumed NAX or PL daily for 8 weeks. No adverse events related to the dietary supplement were reported.

Heart/Brain Axis Assessment
Before and after the supplementation period, subjects performed a VO2max assessment on a treadmill to measure cardiovascular performance (CardioCoach, Korr Medical Technologies). Subjects also completed a psychological mood state survey (POMS, Profile of Mood States) to assess Global Mood State and 6 related subscales: Vigor (V), Tension (T), Depression (D), Anger (A), Fatigue (F), and Confusion (C). The VO2max assessment was designed for participants to reach maximal oxygen consumption and voluntary fatigue within 15 minutes. The protocol
consisted of a gradual warmup of easy walking/jogging, followed by progressive increases in speed and incline each minute until exhaustion. Heart rate (beats per minute, bpm) and oxygen consumption (ml/kg/min) were recorded at maximum and at two submaximal levels (aerobic threshold, AeT and anaerobic threshold, AT). The POMS survey is a self-administered 64-question assessment that takes approximately 20 minutes to complete and was collected before and after the 8-week supplementation period.

**Data Management and Analysis**

All participant data was maintained in a central location and transcribed to a central database. The data was identified by subject number and examined for accuracy and completeness. Tabulated data was analyzed with JMP 8.0 (SAS Institute, Cary, NC) using standard parametric paired t tests, and significance was assessed with a 2-tailed alpha level set at 0.05.

**RESULTS**

Subject baseline characteristics are presented in Table 1. There was no improvement in maximal oxygen uptake (VO2max while running) with NAX supplementation (Figure 1). However, subjects in the NAX group showed a significant ~10% lower average heart rate at submaximal intensities (aerobic threshold and anaerobic threshold) compared to both pre-supplementation values and compared to the placebo group (Figure 2), suggesting a profound “cardiotonic” effect of NAX supplementation with superior metabolic efficiency at submaximal aerobic endurance intensities. Aerobic threshold (AeT) and anaerobic threshold (AT) heart rates were significantly lower post-supplementation of NAX versus PL (AeT; NAX 130±17 v. PL 145±14; and AT; NAX 139±20 v. PL 154±11, p<0.05), indicating that NAX subjects were able to perform a certain level of physical work at a lower relative intensity or higher cardiovascular efficiency.

**Table 1.** Baseline subject characteristics Data represent average (Mean) values (±SD). NAX, natural astaxanthin group; PL, placebo group; VO2max, maximal

|               | NAX        | PL         |
|---------------|------------|------------|
| Height (cm)   | 169 (11)   | 168 (9)    |
| Weight (kg)   | 70.0 (7.1) | 69.3 (7.0) |
| Body Fat (%)  | 20.3 (6.3) | 24.9 (8.4) |
| VO2max (ml/kg/min) | 43.7 (9.1) | 42.9 (7.2) |
| Peak HR (bpm) | 169 (9)    | 172 (9)    |
| AeT VO2 (ml/kg/min) | 33.5 (7.5) | 31.3 (3.8) |
| AeT HR (bpm)  | 145 (13)   | 143 (11)   |
| AT VO2 (ml/kg/min) | 38.5 (8.3) | 36.2 (4.7) |
| AT HR (bpm)   | 154 (15)   | 153 (11)   |

NAX, natural astaxanthin group; PL, placebo group; VO2max, maximal oxygen consumption; Peak HR, heart rate at VO2max; AeT, aerobic threshold; AT, anaerobic threshold.
There was no improvement in maximal oxygen uptake (VO2max while running) with NAX supplementation (Figure 1).

**Figure 1.** Oxygen consumption at baseline (pre-supplementation) and week 8 (post-supplementation). There were no significant differences in absolute oxygen consumption at any time point. NAX, natural astaxanthin group; PL, placebo group; pre, baseline; post, week 8; Peak, maximal oxygen consumption; AeT, aerobic threshold; AT, anaerobic threshold.

However, subjects in the NAX group showed a significant ~10% lower average heart rate at submaximal intensities (aerobic threshold and anaerobic threshold) compared to both pre-supplementation values and compared to placebo (Figure 2), suggesting a profound “cardiotonic” effect of NAX supplementation with superior metabolic efficiency at submaximal aerobic...
endurance intensities. Aerobic threshold (AeT) and anaerobic threshold (AT) heart rates were significantly lower post-supplementation in NAX versus PL (AeT; NAX 130±17 v. PL 145±14; and AT; NAX 139±20 v. PL 154±11, p<0.05), indicating that NAX subjects were able to perform a certain level of physical work at a lower relative intensity or higher cardiovascular efficiency.

In addition, significant improvements were found in NAX for some positive mood state parameters (Figure 3): Global Mood (+11%, p<0.05) & Vigor (+5%, NS); and some negative mood state parameters: Tension (-20%, NS), Depression (-57%, p<0.05), Anger (-12%, NS), Fatigue (-36%, p<0.05), and Confusion (-28%, NS), indicating a beneficial psychological effect of NAX on overall mood and specifically in reducing depression and fatigue.

**Figure 3.** Psychological Mood State at baseline (pre-supplementation) and week 8 (post-supplementation).

**Figure 3a.** Improvements were found in NAX for Global Mood (-11%, NAX=127+20 v. PL=127+20; p<0.05)* *Global Mood State = a lower score indicates a more positive psychological mood state.

**Figure 3b:** Improvements were found in NAX for some mood state subscales: Tension (-20%, NS), Depression (-57%, p<0.05), Anger (-12%, NS), Vigor (+5%, NS), Fatigue (-36%, p<0.05), and Confusion (-28%, NS).
DISCUSSION

Astaxanthin (AX) is a naturally occurring carotenoid, synthesized primarily by marine microalgae, with powerful antioxidant and anti-inflammatory properties. In mammals, dietary AX accumulates in muscle, where it attenuates muscle damage and inhibits peroxidation of DNA and lipids due to prolonged exercise [18-20]. In addition, AX has been identified as a nutrient that may strongly stimulate fat oxidation during exercise. AX supplementation of mice (4-5 weeks with 6-30mg/kg BW) improves fat utilization and increases swimming and treadmill running time to exhaustion [20, 21]. These effects were theorized to be attributable to an improved mitochondrial capacity for fatty acyl-CoA uptake via an improvement in carnitine palmitoyltransferase 1 (CPT1) function, subsequent to inhibition of oxidative damage to the mitochondrial membrane. Such pre-clinical results provide suggestive evidence that AX may have potential ergogenic effects for endurance athletes.

Previous studies of NAX administration in animal models have shown a decrease in exercise-induced damage to skeletal and cardiac muscle, as well as an increase in redox balance, fat oxidation and time to exhaustion during exercise [18-23]. Some positive rodent studies have administered NAX at fairly high doses of 6-30mg/kg [19-22], while others have used lower amounts (1mg/kg) to delay physical exhaustion and improve redox balance [24] - relatively higher than the amounts of NAX supplemented in human trials (2-20mg/day).

Humans studies of NAX supplementation have noted improved antioxidant status as well as reduced oxidative damage in sedentary obese subjects [26, 27] and untrained men [28]. In athletes, NAX supplementation for 4 weeks reduced lactic acid accumulation following 1200m of running [23]. Earnest et al. [25] found significant improvements in power output (+15% = 20W mean power increase) and faster completion of a 20km cycling time trial (5% = 2min mean change) following NAX supplementation (4mg/day for 4 weeks).

Jiang et al [29] recently demonstrated the anti-depressant effects of NAX, subsequent to serotonergic and anti-inflammatory effects, in an animal model of stress-induced depression. These and other authors suggest that NAX may be advantageous in neuroprotection due to its ability to locate inside the phospholipid membrane and at the membrane surface, as well as its facility in crossing the blood-brain barrier [29-32], which can lead to a wide range of potential psychological benefits including enhanced cognitive function [33, 37, 39], reduced depression [33], lower anxiety [35, 36], and neuroprotection [39, 40].

The current study found intriguing psychological mood state benefits as well as physical cardiotonic benefits of NAX supplementation. At submaximal exercise intensities, supplementation with 12mg/day of NAX for 8 weeks significantly reduced heart rate at the same relative workload at aerobic threshold (AeT) and anaerobic threshold (AT). Submaximal exercise heart rates were ~10% lower following NAX supplementation, where average heart rates were ~130-145 bpm (aerobic threshold) to ~139-154 bpm (anaerobic threshold), but not at higher “peak” intensities (e.g. ~165-172 bpm at peak VO2max). Combined with promoting significantly higher overall mood (Global Mood State) and reduced depression and fatigue, NAX supplementation delivered intriguing support of the psychophysiological axis between the brain and the heart.

CONCLUSIONS

NAX supplementation lowered average heart rate at submaximal endurance intensities (suggesting a “physical” heart benefit) and improved mood state parameters (suggesting a “mental” brain
benefit). While previous studies have demonstrated how NAX supplementation can improve parameters associated with heart health (antioxidant, fat oxidation, endurance) and brain health (neuro-inflammation, cognition, antidepressant/anxiolytic), these results are the first to show that natural astaxanthin supplementation supports the psychophysiological “heart-brain-axis” with simultaneous improvements in both physical and mental wellness.

In addition to these “psychophysiological” benefits for the heart/brain axis, these results suggest that NAX supplementation may also be a beneficial ergogenic aid for long-distance and ultra-distance endurance athletes (e.g. marathon runners, Ironman triathletes, and ultra-runners/cyclists), where both physiological endurance and psychological balance are simultaneously stressed and the support of both may be a novel approach to improving physical/mental performance.

Because of the dual nature of NAX benefits on heart and brain health, future studies are warranted in expanded populations including elderly subjects and those with cardiac and neurologic complications including post-myocardial infarction, heart failure, statin usage, mitochondrial dysfunction, chronic fatigue, and related conditions.

**List of Abbreviations:** NAX: natural astaxanthin; POMS: Profile of Mood States; VO2max: maximal oxygen consumption; AeT: aerobic threshold; AT: anaerobic threshold

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**Disclosure**
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Astaxanthin Supplementation Reduces Depression and Fatigue in Healthy Subjects

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Abstract

Objective: Natural Astaxanthin from Haematococcus pluvialis microalgae (NAX) has been researched in hundreds of clinical trials, pre-clinical animal studies and in-vitro surveys for various bioactive properties that indicate potential preventive and therapeutic health benefits. Among the most widely-researched properties of astaxanthin in the literature are broad-spectrum anti-inflammatory activity and powerful antioxidant capacity. In addition, both human and animal research have revealed a wide range of potential benefits for neurological and eye health, cardiovascular function, exercise endurance, enhancement of the immune response and skin health. This study’s goal was to explore the effects of a daily dose of 12 mg per day of NAX on psychological mood state in healthy subjects.

Methods: This study employed placebo control and parallel design under double blind conditions. A total of 28 healthy subjects, half male and half female, with a median age of 42, supplemented with 12 mg per day of NAX or placebo. Before Day 0 and again at the end of the 8-week supplementation period, subjects completed a validated Profile of Mood States (POMS) survey to assess global mood state (GM) and related subscales: Vigor (V), Tension (T), Depression (D), Anger (A), Fatigue (F) and Confusion (C).

Results: Significant improvements were found in the NAX treatment group for positive mood state parameters: GM (+11%, p < 0.05) and V (+5%, NS); and negative mood state parameters: D (-57%, p < 0.05), F (-36%, p < 0.05), T (-20%, NS), A (-12%, NS), and C (-28%, NS).

Conclusions: While previous studies have shown NAX supplementation to improve parameters associated with brain health (neuro-inflammation and cognition), these data are the first to suggest that natural astaxanthin supplementation reduces negative mood state parameters (depression and fatigue) and improves global mood state and thus supports mental wellness.

Keywords: Antioxidant; Astaxanthin; Carotenoid; Cardiovascular; Mood State; Mental Wellness

Abbreviations

NAX: Natural Astaxanthin; POMS: Profile of Mood States; GM: Global Mood State; D: Depression; T: Tension; A: Anger; F: Fatigue; V: Vigor; C: Confusion; BW: Body Weight

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Introduction

Natural astaxanthin (NAX) is a red carotenoid that provides characteristic color to pink flamingo feathers, red shrimp shells, and pink salmon flesh. The high level of NAX in these tissues is reflective of the animal's dietary intake of NAX from microalgae, copepods, krill, and other lower food chain organisms [1,2]. Astaxanthin is found in the human food chain in red-hued sea animals including crab, shrimp and lobster (in low quantities) and in the highest quantity in wild salmon [3-5]. NAX exists as one stereoisomeric form (3S,3’S) [2]. Other stereoisomeric forms of astaxanthin exist in yeast and bacteria as well as in astaxanthin produced synthetically from petrochemicals (SAX). The other forms found in these alternative sources are (3R,3’R), and (3R,3’S) (meso). Astaxanthin synthesized from petrochemicals is widely used in farmed fish such as salmon and trout. Synthetically produced astaxanthin contains all three stereoisomeric forms while wild salmon contains exclusively 3S,3’S [6]. NAX is found in nature in the highest concentration in Haematococcus pluvialis microalgae which is the source of the vast majority of astaxanthin consumed in supplement and functional food forms. NAX is widely accepted by regulatory bodies around the world as a human nutritional supplement and in functional foods. Other forms including SAX and forms from yeast and bacteria are generally not accepted by regulators or are accepted with restrictions due to lack of safety data and clinical research in humans. NAX contains small amounts of related carotenoids naturally occurring in the microalgae and is primarily esterified with fatty acid molecules attached at the end of the astaxanthin molecule. 3S,3’S stereoisomers comprise approximately 96% of the carotenoid fraction. The additional carotenoids found in NAX include zeaxanthin, lutein, canthaxanthin and beta-carotene. These chemical differences between forms of astaxanthin and the co-existence of other natural carotenoids may result in increased bioavailability and synergistic benefits for NAX as compared to SAX [7,8].

NAX has been studied for its antioxidant, anti-inflammatory, eye health benefits, cardioprotective properties, immune system modulatory activity and neuroprotective activities in humans [9-13]. Studies in rats and mice have indicated that NAX may reduce hypertension and increase blood flow [14,15]. The mechanisms proffered in this research were a modulatory effect on cellular stress pathways such as nitric oxide, nuclear factor E2 related factor 2 (Nrf2) and nuclear factor kB (Nfkb) [16].

Astaxanthin has demonstrated wide-ranging benefits for athletes including improving endurance and performance, decreasing lactic acid levels after exercise and increasing fat oxidation/metabolism [17-27]. Dietary AX accumulates in muscle tissue in mammals. In muscles heavily worked during prolonged exercise, it prevents peroxidation of DNA and lipids and reduces muscle damage [17-19]. Furthermore, during exercise, AX may ameliorate fat oxidation. Mice supplemented with AX at 6 - 30 mg/kg BW over 4 - 5 weeks demonstrated increased fat utilization and longer treadmill running time/swimming time before exhaustion [19,20]. The mechanism for these effects was postulated as improvement in mitochondrial capacity of fatty acyl-CoA uptake through improvement of the function of carnitine palmitoyltransferase 1 (CPT1), which occurs due to astaxanthin’s protective effect of the mitochondrial membrane from oxidative damage. Earlier research on NAX’s effects in animal models demonstrated attenuation of exercise-induced damage to cardiac and skeletal muscle, along with improvement in fat oxidation, redox balance and time to exhaustion during exercise [17-22]. Several rodent studies supplemented NAX at 6 - 30 mg/kg which would translate to high dosages in humans [18-21]. Another rodent study employed a significantly lower dosage of 1 mg/kg and still found increased time to physical exhaustion and improvement in redox balance [23]. (Human trials have found positive results for a variety of health benefits at much lower dosages per kg BW than the rodent studies in the literature. The normal range in human clinical research has been 2 mg to 20 mg per day regardless of BW.) Many of astaxanthin's effects may be attributable to a hypothesized mitochondrial-centric mechanism, which could improve energy and redox cellular metabolism (e.g. via Nrf2-ARE pathway activation). Indeed, mitochondrial redox metabolism has been implicated in various neurological disorders including Alzheimer's, Parkinson's, and age-related dementia, so it is logical that NAX has been suggested to play a putative prophylactic role in cardiovascular as well as neurologic conditions [28-39].

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Antioxidant status showed consistent improvement in human research on obese subjects leading a sedentary lifestyle [25,26] as well as in untrained men [27]. Athletes supplementing with NAX for 4 weeks experienced reduced lactic acid levels after running 1200 meters [22]. In a study on competitive cyclists, significant improvements in power output (15%) and improvement in cycling time trial (5% reduction over 20 km for an average 2 minute mean decrease) was found at a relatively low dosage of 4 mg per day over 4 weeks [24].

**Material and Methods**

**Participants**

Twenty-eight healthy, active, non-depressed adult subjects (14 men and 14 women) were supplemented with NAX for 8-weeks (age 42 ± 8, range 26 - 63 years; height 169 ± 10 cm; BW 69 ± 6 kg). An external ethics board reviewed informed consent forms which all subjects completed prior to commencement of the study.

**Astaxanthin source**

Using a double-blind method, the subjects were randomly separated into two pools. The treatment group received a NAX supplement (AstaZine® Natural Astaxanthin, BGG/AlgaeHealth Sciences) while the control group received identically-matching placebo (PL).

The NAX supplement contained an extract of *Haematococcus pluvialis* containing 12 mg of astaxanthin which was combined with medium chain triglyceride oil and a small amount of d-alpha tocopherol (10 IU) to maintain stability of the capsule formulation. Supplementation of NAX or PL continued by all subjects each day for the duration of the 8-week study period.

No adverse events related to ingestion of NAX or PL were reported.

**Mood state assessment**

Before and after the supplementation period, subjects completed a validated psychological mood state survey (POMS, Profile of Mood States) to assess Global Mood State and 6 related subscales; Vigor (V), Tension (T), Depression (D), Anger (A), Fatigue (F), and Confusion (C). The POMS survey is a self-administered 64-question assessment that takes approximately 20 minutes to complete and was collected before and after the 8-week supplementation period.

**Data management and analysis**

A database was employed in a central location to maintain participant data. Subject numbers were used to identify the data, with thorough examination for completeness and accuracy. JMP 8.0 (SAS Institute, Cary, NC) using standard parametric paired t tests was employed to analyze the tabulated data, with a 2-tailed alpha level set at 0.05 used to assess significance.

**Results**

Baseline characteristics for participants are presented in table 1. Significant improvements were found in NAX for positive mood state parameters (Figures 1 and 2): Global Mood (+11%, p < 0.05) and Vigor (+5%, NS); and negative mood state parameters: Depression (-57%, p < 0.05), Fatigue (-36%, p < 0.05), Confusion (-28%, NS), Tension (-20%, NS), and Anger (-12%, NS) indicating a beneficial psychological effect of NAX on overall mood and specifically in reducing depression and fatigue.

|                | NAX   | PL    |
|----------------|-------|-------|
| Height (cm)    | 169(11)| 168 (9) |
| Weight (kg)    | 70.0 (7.1)| 69.3 (7.0) |
| Body Fat (%)   | 20.3 (6.3)| 24.9 (8.4) |

*Table 1: Baseline subject characteristics. Data represent average (Mean) values (±SD). Abbreviations: NAX: Natural Astaxanthin Group; PL: Placebo Group.*

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**Figure 1:** Psychological Global Mood State at Baseline (pre-supplementation) and week 8 (post-supplementation). Improvements were found in NAX for Global Mood (-11%, NAX = 127±20 v. PL = 127±20; p < 0.05)*

*Global Mood State = a lower score indicates a more positive psychological mood state.

Abbreviations: NAX: Natural Astaxanthin Group; PL: Placebo Group.

**Figure 2:** Psychological Mood State Sub-Scales at Baseline (pre-supplementation) and week 8 (post-supplementation). Improvements were found in NAX for some mood state subscales: Tension (-20%, NS), *Depression (-57%, p < 0.05), Anger (-12%, NS), Vigor (+5%, NS), *Fatigue (-36%, p < 0.05), and Confusion (-28%, NS).

Abbreviations: NAX: Natural Astaxanthin Group; PL: Placebo Group.
Discussion

Jiang, et al. [28] recently demonstrated potential psychological effects of NAX, subsequent to serotonergic and anti-inflammatory effects, in an animal model of stress-induced depression. Other animal models suggest that NAX may be advantageous in neuroprotection due to its ability to locate inside the phospholipid membrane and at the membrane surface, as well as its facility in crossing the blood-brain barrier [28-31] leading to a wide range of potential psychological benefits including enhanced cognitive function [32,36,38], reduced depression [32], lower anxiety [34,35] and neuroprotection [38,39].

The heart is recognized to play a vital role not only as a circulatory pump, but as part of a psychophysiological network as a generator and transmitter of system-wide information throughout multiple body systems, including the nervous system [40-45]. Electrical input from the heart can dynamically influence homeostatic, cognitive, perceptual, and emotional processing in the brain, thus having the potential to affect myriad aspects of mood and behavior [40-43]. Thayer and Lane [40] reviewed the direct and indirect connections between the heart and the brain, including the physiological, behavioral, emotional, and cognitive processes involved in bi-directional control of cortical/cardiac function. Organ crosstalk between the brain/heart has been noted in stress-related cardiomyopathy syndromes and traumatic brain injury [41] including the use of heart rate variability (HRV) for its importance in gauging both the state of the heart (physical stress) as well as the state of the brain (psychological stress). Yoga, as an intervention to restore balance heart-brain crosstalk through plasticity and stability of the autonomic nervous system, has been used to reduce anxiety levels, atrial fibrillation episodes, blood pressure and neurocardiogenic syncope [42]. Studies have also shown that positive emotional states may improve function of both the cardiovascular and immune systems [44,45].

Human clinical research on astaxanthin’s effects on brain health have shown some promising potential health benefits. In a trial on elderly subjects with age-related forgetfulness, improvement in age-related decline in cognitive and psychomotor function was found in the treatment group when supplementing with 12 mg per day of NAX over 12 weeks [46]. A subsequent study measured a marker for dementia, phospholipid hydroperoxides, which accumulate in the erythrocytes of dementia patients. The researchers tested 6mg of NAX and 12 mg of NAX against placebo over 12 weeks. At both dosages, NAX significantly reduced the levels of this dementia marker [47]. Lastly, a study testing for cognitive function of healthy elderly subjects who complained of age-related forgetfulness was done over 12 weeks. The subject pool was 96 people randomly assigned to take 6 mg of NAX, 12 mg of NAX or placebo. A variety of tests were done on these subjects including blood work, urine screens and assessments on two different cognitive tests called the Groton Maze Learning Test and CogHealth. Results in both the 6 mg and 12 mg treatment groups were significant, with slightly better results in the 12 mg group. The researchers concluded that NAX improves cognitive function in healthy aged subjects [48].

The current study found intriguing psychological mood state benefits of NAX supplementation. The results were in a different area of mental health as compared to the three clinical trials cited immediately above, which examined potential benefits for the brains of an aging population. The results in this present study may apply to a general population regardless of age. They indicate that people may feel better mentally by supplementing with NAX. Results leading to this hypothesis include a significantly higher overall mood (Global Mood State +11%, p < 0.05) and a significant reduction in Depression (-57%, p < 0.05) and Fatigue (-36, p < 0.05). This was the first study to demonstrate these results in a population of healthy human volunteers.

Conclusions

While previous studies have shown NAX supplementation to improve parameters associated with brain health (neuro-inflammation and cognition), these data are the first to suggest that natural astaxanthin supplementation reduces negative mood state parameters (depression and fatigue) and improves global mood state and thus supports mental wellness.
Acknowledgments

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Disclosure

This work was funded by Algae Health Sciences, a division of BGG. Talbott was principal investigator and reports no conflicts of interest in this work. Hantla reports no conflict of interest. Capelli is an employee of Algae Health Sciences, and Li, Ding and Artaria are employees of BGG, BGG North America and BGG Europe, which are companies involved in the production and distribution of NAX.

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**Abstract:** (1) Background: Reperfusion injury refers to the cell and tissue damage induced, when blood flow is restored after an ischemic period. While reperfusion reestablishes oxygen supply, it generates a high concentration of radicals, resulting in tissue dysfunction and damage. Here, we aimed to challenge and achieve the potential of a delivery system based on astaxanthin, a natural antioxidant, in attenuating the muscle damage in an animal model of femoral hind-limb ischemia and reperfusion. (2) Methods: The antioxidant capacity and non-toxicity of astaxanthin was validated before and after loading into a polysaccharide scaffold. The capacity of astaxanthin to compensate stress damages was also studied after ischemia induced by femoral artery clamping and followed by varied periods of reperfusion. (3) Results: Histological evaluation showed a positive labeling for CD68 and CD163 macrophage markers, indicating a remodeling process. In addition, higher levels of Nrf2 and NQO1 expression in the sham group compared to the antioxidant group could reflect a reduction of the oxidative damage after 15 days of reperfusion. Furthermore, non-significant differences were observed in non-heme iron deposition in both groups, reflecting a cell population susceptible to free radical damage. (4) Conclusions: Our results suggest that the in situ release of an antioxidant molecule could be effective in improving the antioxidant defenses of ischemia/reperfusion (I/R)-damaged muscles.

**Keywords:** astaxanthin; ischemia/reperfusion injury; reactive oxygen species; oxidative stress; cyclodextrin

1. Introduction

An ischemic condition results in an imbalance in the oxygen production and consumption in the cells and tissues, generating a constant influx of pro-inflammatory reactions that render tissues vulnerable to microvascular dysfunction [1] and to additional injury [2]. Consequently, an oxidative stress status is generated, characterized by an overconcentration of reactive oxygen species (ROS) [3]. An increase of tissue injury and high inflammatory response [4] have been associated with the reperfusion process due to the endogenous antioxidant defense system’s inability to handle the radical
load and restore the affected environment [5,6]. In the case of lower limbs where muscles represent the primary mass of tissue [7], damage triggered by ischemia/reperfusion (I/R) represents the most critical effect of the inflicted injury. According to Gardner et al. [8], antioxidant and anti-inflammatory strategies may be useful to treat lower I/R pathologies. Moreover, animal studies have demonstrated the efficacy of antioxidant therapy in preventing or attenuating the I/R injury [2,9].

Natural carotenoids have shown particular antioxidant capabilities to scavenge ROS and enhance the cell’s ability to prevent oxidative stress [10]. Astaxanthin, a xanthophyll carotenoid, has been studied for its antioxidant and anti-inflammatory properties [11]. Pretreatment with oral astaxanthin supplements has shown to reduce oxidative stress and inflammation in rodents presenting ischemic renal injuries [12], and to reduce apoptosis and autophagy in animals with hepatic ischemia [13]. Furthermore, the oral ingestion of either astaxanthin or vitamin E in myocardial I/R injury for 21 days showed a higher cardioprotection when treated with astaxanthin [14]. Intracerebroventricular injection of astaxanthin revealed a reduction in ischemia-related injury in brain tissue through the inhibition of oxidative stress, reduction of glutamate release, and anti-apoptosis [15]. Apart from these positive results, some drawbacks regarding in vivo stability and bioavailability of astaxanthin have also been reported [16]. A strategy based on the use of carriers for in situ delivery of astaxanthin represents a possible solution to enhance its in vivo effect. We previously showed that hydroxypropyl-β-cyclodextrin-astaxanthin (CD-A) complexes allow the stabilization and preservation of astaxanthin activity. CD-A showed the protection of human endothelial cells under exogenous oxidative stress [17]. In this study, we first challenged the efficacy of a polysaccharide system for the in situ delivery of CD-A. Two natural polysaccharides, pullulan and dextran, were selected to produce scaffolds. This choice was supported by previous works, which showed good biocompatibility, biodegradability, and flexibility of pullulan and dextran in the cardiovascular field, when used as delivery systems [18–21]. Then, the preservation of antioxidant activity of astaxanthin after being loaded into the scaffolds and the non-toxicity of the system were confirmed. Finally, an animal model of I/R injury was selected as the proof of concept. The occlusion of the femoral artery was chosen, since the superficial femoral and popliteal arteries are continuously affected by ischemic and reperfusion periods [22]. Astaxanthin capacity to compensate muscle damages was determined after 45 min of ischemia induced by femoral artery clamping and followed by varied periods of reperfusion (60 min, 7 or 15 days), as compared with the sham (I/R muscles without antioxidant treatment) and control groups. The gracilis muscle was explanted and histologically evaluated to assess tissue response regarding inflammatory cell infiltration, absence of toxicity, and indirect oxidative stress status.

2. Results

2.1. P/D/CD-A Scaffold Loading and Antioxidant Activity Evaluation

Environmental scanning electron microscopy (ESEM) images reflected a heterogeneous microporous structure of the pullulan/dextran (P/D)-based scaffold (Figure 1A,B), spread all over the surface. Interestingly, the addition of the CD-A within the P/D scaffold smoothed the scaffold pores (Figure 1B). As presented in Figure 1C, the freeze-drying process had an easy handle to the scaffold, as well as a high and fast molecule-loading capacity. Five minutes were required for the complete absorption of the molecule (10 μL/scaffold). The volume needed to charge the scaffold was determined as the swollen maximum capacity without volume saturation (data not shown). The initial dimension of the scaffold was reduced after the complete absorption of different conditions, varying from 5 × 5 mm to 3 × 3 mm.
Figure 1. Environmental scanning electron microscopy (ESEM) of the (A) pullulan/dextran (P/D) scaffold and (B) P/D/hydroxypropyl-β-cyclodextrin-astaxanthin (CD-A) scaffold. (C) P/D scaffolds were loaded with 10 µL of either saline solution or CD-A until complete absorption. (D) CD-A release kinetics from the P/D scaffolds during 60 min in PBS (pH 7.4). The results indicate mean ± SD (n = 46). (E) Antioxidant activity evaluation of CD-A and P/D/CD-A release product at 12.5 µM compared to Trolox at 12.5 µM standard antioxidant; results are presented as area under the curve (AUC) net values; the results are expressed as mean ± SD (n = 46); values connected with the same letter (a, b) are not significantly different considering p < 0.05 as the significance level.

P/D/CD-A release product was quantified regarding its concentration and antioxidant capacity. First, a calibration curve using different concentrations of CD-A (0–12.5 µM astaxanthin concentration) versus absorbance at 472 nm was plotted (Figure S1A), then the P/D scaffold was charged with the maximum amount used for the CD-A quantification (2.5 mg; astaxanthin concentration 12.5 µM) and P/D/CD-A controlled kinetics release was reported. Figure 1D indicates a fast release during the first 20 min (50%) and a total release after 60 min. Then, calibration curves using Trolox (standard antioxidant, 0–50 µM) and CD-A (0–50 µM) were plotted versus the area under the curve (AUC) net (Figure S1B). Slope ratio from both curves indicated a high antioxidant capacity of CD-A when
compared to the standard antioxidant (2.8 mM Trolox Equivalent Antioxidant Capacity (TEAC)). Figure 1E shows a lower AUC net value for CD-A release product (11.2) against 12.7, the expected value for a total CD-A release; thus, indicating a slight retention of the CD-A antioxidant activity by the P/D scaffold. However, when analyzing all antioxidants at the same concentration, CD-A alone or as a release product, both presented a higher antioxidant activity than Trolox, the reference antioxidant molecule.

2.2. Evaluation of In Vitro and In Vivo Toxicity of P/D/CD-A Scaffold

P/D-based scaffolds were autoclaved by UV light and evaluated to determine any sign of in vitro and in vivo toxicity. The toxicity of CD-A and P/D/CD-A release product (12.5 µM) was evaluated after contact with the 3T3 cells for 24 h. This cell line allows the evaluation of toxicity of samples thanks to its high sensitivity. Figure 2 shows an acceptable cell viability (superior to 70% of negative control (NC)), when 3T3 cells were incubated with CD-A (0–5 µM) or with CD-A release products.

![Cell viability assay](image)

**Figure 2.** Cell viability assay of CD-A and P/D/CD-A release product by MTT assay. Antioxidant samples were significantly different compared to the positive toxicity control (PC). The results indicate mean ± SD (n = 3).

In vivo toxicity was evaluated by monitoring the response of vital organs distant from the implant. No noticeable systemic reaction was triggered by the presence of either the P/D scaffold or the P/D/CD-A antioxidant in the kidney and liver (Figure S2) of the animals subjected to ischemia (45 min) and reperfusion (7 or 15 days) compared to the control group.

2.3. In Vivo Evaluation of P/D/CD-A Scaffolds in Femoral I/R Model

2.3.1. Histological and Immunohistological Analysis

During the in vivo implantation study (Figure 3), animals did not present any alteration in their normal behavior or any dietary changes that would reflect suffering. No sign of motion restriction or external acute inflammation was observed when the entire I/R protocol was being followed. After 45 min of ischemia, artery blood flow was gradually restored in the sham and antioxidant groups (confirmed by patency test) in different reperfusion periods (60 min, 7 or 15 days). P/D scaffolds remained in the site of implantation in both P/D (sham) and P/D/CD-A (antioxidant) groups, even after 15 days of analysis, allowing the delivery of the astaxanthin molecule from the scaffold to the surrounded I/R area.
Figure 3. In vivo experimental design and surgical ischemia/reperfusion (I/R) procedure. (A) The study animals were assigned into three different groups: control \((n = 12)\), sham \((n = 12)\), and antioxidant \((n = 12)\). (B) Control animals underwent 2 h of general anesthesia. The sham and the antioxidant groups underwent 45 min of ischemia induced by the ligature of the femoral artery. Then an intramuscular incision, perpendicular to the muscle fibers, was made in the gracilis muscle, allowing the implantation and keeping hold of the P/D scaffold charged with saline solution in the sham group or the P/D scaffold loaded with the antioxidant molecule \((P/D/CD-A)\) in the antioxidant group, before artery reperfusion for either 60 min, 7 or 15 days. Finally, the gracilis muscles were explanted and histologically evaluated. Hematoxylin eosin staining (Figure 4) of the control muscles showed a normal morphology with the grouped fibers in a fascicular pattern (Figure 4A). Conversely, P/D and P/D/CD-A groups that were subjected to I/R injury presented muscle fibers with a mild hypertrophic aspect, particularly after 7 and 15 days of reperfusion compared to the control fibers (Figure 4B,C). A fibrous capsule (yellow arrows) that surrounded all P/D-based scaffold-tissue interfaces after 7 and 15 days of reperfusion, was also observed. The thickness of this fibrous capsule was similar in both P/D and P/D/CD-A groups, subjected to the same reperfusion periods.
Figure 4. Hematoxylin eosin staining of gracilis muscle of (A) control, (B) sham, and (C) antioxidant groups. The sham and the antioxidant groups that underwent 45 min of occlusion of the femoral artery, were followed by 60 min, 7- or 15-day reperfusions, and treated with P/D scaffold charged with saline solution (sham group) or CD-A (antioxidant group). Scaffolds are stained in blue and surrounded by red dashed lines; gracilis muscle are stained in pink; the fibrous capsule is indicated by yellow arrows. Scale bars, 200 µm; magnification 10×. Experiences were realized in three independents experiments ($n = 4$ animals per group).

2.3.2. Phagocyte Responses to Scaffold

Monocytes/macrophages are phagocytic cells consisting of two cellular subtypes: (i) M1 related to the inflammatory process and positively labeled with the anti-CD68 antibody; (ii) M2 related to the tissue regeneration process and showing positive labeling with anti-CD68 and anti-CD163 antibodies. Evaluation after 60 min of reperfusion demonstrated no significant inflammatory response given by positive CD68 or CD163 markers in any of the I/R groups. An example of histological images is presented in Figure 5A for CD68 positive detection. A significant difference ($p < 0.05$) was found in the total CD68 monocyte/macrophage positive population at 7 days of reperfusion between the P/D and the antioxidant-loaded scaffold P/D/CD-A (Figure 5A, Figure S4A), being almost two times higher according to the image quantification. After 15 days of reperfusion, a non-significant difference was achieved between I/R groups by a slight increase in the P/D CD68 positivity. Few cases of P/D/CD-A scaffolds presented cell colonization composed of CD68 positive marked cells, observed after 15 days of implantation. CD163 positive cells were observed surrounding the scaffold, as well as the gracilis muscle’s periphery, where the scaffold was present (Figure 5B, Figure S4A). After 60 min, a significant difference ($p < 0.05$) was observed between control, P/D, and PD/CD-A samples despite the lower values registered for all three samples. Compared to the sham group, there were no significant changes in the number of cells with positive staining for the CD163 marker after 7 days of reperfusion in the
P/D/CD-A group. However, a significant increase of M2 repair macrophages ($p < 0.05$) was observed in the P/D/CD-A group after 15 days.

(Figure 5B, Figure S4A). After 60 min, a significant difference ($p < 0.05$) was observed between control, P/D, and PD/CD-A samples despite the lower values registered for all three samples. Compared to the sham group, there were no significant changes in the number of cells with positive staining for the CD163 marker after 7 days of reperfusion in the P/D/CD-A group. However, a significant increase of M2 repair macrophages ($p < 0.05$) was observed in the P/D/CD-A group after 15 days.

Figure 5. Immunohistochemical detection of monocyte/macrophage populations by CD68 and CD163 markers. Immunological staining and quantification of gracilis muscles containing P/D implanted or P/D/CD-A scaffolds using (A) CD68 and (B) CD163 markers. In all the cases, the muscle was subjected to 45 min ischemia and reperfused during 60 min, 7 or 15 days. The quantification was done using Matlab and contrasted with the pathologist analysis. The results indicate mean ± SD ($n = 6$). * $p < 0.05$ P/D samples versus the control group and ** $p < 0.05$ PD/CD-A samples versus the control group used to define statistical significance. Red lines indicate the scaffold localization. Scale bars: 200 μm/10× magnification. Experiences were realized in three independent experiments (4 animals per group).
2.3.3. Nrf2/HO1/NQO1 Endogenous Antioxidant Systems

The effect of CD-A local treatment on nuclear factor-erythroid 2-related factor 2 (Nrf2) translocation in the gracilis muscle was studied under I/R conditions. Nrf2 is a crucial transcription factor, which regulates antioxidant defense comprising antioxidant enzymes heme oxygenase-1 (HO1) and NAD (P) H: quinone oxidoreductase 1 (NQO1) [23]. Regarding this, the expression of NQO1 and HO1 proteins was also evaluated. The immunohistochemical detection showed a positive phosphorylated Nrf2 nuclear staining at 7 and 15 days of reperfusion in the P/D/CD-A group (Figure 6A; antibody positive control in a rat and mouse heart is presented in the supplementary data, Figure S3). The quantitative analysis reflected a positive staining which gradually went along with the increased reperfusion periods in both groups (Figure 6A, Figure S4B). It is worth noting that even after 15 days, the P/D/CD-A group showed less Nrf2 positivity compared to the sham group wherein Nrf2 expression was more pronounced. Nonetheless, the variability of the responses between both groups did not lead to significant differences. The expression levels of HO1 and NQO1 were evaluated on the gracilis muscle under I/R conditions after 15 days of reperfusion. After this period, we observed no enhancement in HO1 expression by immunohistochemical staining, neither in sham nor in P/D/CD-A groups. However, we observed a lower NQO1 protein labeling in the P/D/CD-A group compared to the sham (Figure 6B).

![Image of Figure 6](image_url)

**Figure 6.** Nuclear factor-erythroid 2-related factor 2 (Nrf2), NAD (P) H: quinone oxidoreductase 1 (NQO1), heme oxygenase-1 (HO1) and Perls/DAB (3,3’-diaminobenzidine) detection in the gracilis muscle loaded with P/D or P/D/CD-A scaffolds at different reperfusion periods. Immunohistochemical detection and quantification of (A) Nrf2 (B) NQO1, HO1, and (C) Perls/DAB, which were evaluated for positive retention of iron in the proximal region of the scaffold by the red blood cells. The quantification of Nrf2 and Perls/DAB was done using Matlab and contrasted with the pathologist’s analysis. The results indicate mean ± SD (n = 6). *p < 0.05 P/D samples versus the control group and **p < 0.05 PD/CD-A.
samples versus the control group used to define statistical significance. Red lines indicate the scaffold localization. Scale bars: 200 μm/10× magnification. Experiences were realized in three independent experiments (4 animals per group).

2.3.4. Detection of Oxidative Stress induced by Iron Overload in the Tissues

A positive tissue staining with Perls/DAB (3,3’-diaminobenzidine) was differentiated from red blood cells, heme or non-heme iron fixation. After 60 min and 7 days of reperfusion, the P/D/CD-A group showed a decrease in the Perls/DAB positive staining compared to the sham group. However, after 15 days of reperfusion, a slight increase in the non-heme iron fixation in the muscle area next to the implanted scaffold in either the sham or the antioxidant group suggested a retention by macrophages. Positive heme iron fixations of the red blood cells were noticed in all the groups (Figure 6C) and a negative expression was found when Perls was not amplified by the DAB component in all conditions (Figure S4C), contrasting with the positive stain observed after DAB staining attributed to the presence of red blood cells (heme iron staining). Nevertheless, any retention in the gracilis muscle or the surrounded scaffold was found using both Perls or DAB techniques independently in all the groups (Figure S2C, Figure S4C).

3. Discussion

As the main results of this study, a polymeric system composed of a mixture of two polysaccharides, pullulan and dextran, might allow the delivery of an antioxidant molecule such as astaxanthin in an easy and controlled manner. This system could be used as an in situ antioxidant treatment modality to enhance the muscle damage induced by I/R injury.

In this study, the I/R protocol consisting of the femoral artery clamping in the section between the popliteal and the deep femoral artery during 45 min was chosen. This model was described as the most suitable model of chronic mild ischemia and the most representative of the degree of ischemia seen in patients [24]. Under lower limb I/R conditions, the peripheral muscles are subjected to environmental alterations produced by a higher ROS influx which leads to the depression of the inner body defense mechanism, inducing an imbalance between a burst of ROS and the inability of re-oxygenated cells to handle this radical load, leading to cell degeneration process [5], thereby disturbing their redox stability. Moreover, although muscular damages are induced after ischemic periods, re-establishment of circulation after reperfusion represents a major alteration in the morphological structure [25].

A growing body of literature has reported that after 60 min of reperfusion, a muscular lesion is induced [26–28].

Previous studies have reported the capacity of antioxidants to attenuate oxidative stress after hind-limb I/R injury [29–33]. Particularly, several studies have evaluated the antioxidant effect of astaxanthin in I/R models via oral and intravenous administration pathways [13,34–36]; nevertheless, limitations regarding the biodistribution and stability of the molecule once inside the human or animal body must have been considered. In this study, the in situ delivery of astaxanthin to the site of interest in a controlled manner using a biocompatible scaffold system was intended to address these limitations. The polymer network used in this study was formed through phosphoester linkages between pullulan and dextran by sodium trimetaphosphate (STMP) chemical cross-linking, and scaffold porosity was achieved by the addition of sodium chloride [18]. The fact that the implanted scaffolds remained in the site of implantation within the gracilis muscle even after 15 days of implantation confirmed the feasibility of using this system for the in situ delivery of an antioxidant molecule during a specific period of time. These results corroborate those of a previous work that showed the preservation of the scaffold after 30 days of incubation in a simulated physiological environment [18].

Further, the biocompatibility of P/D scaffolds has been widely evaluated in vitro and in vivo. No negative side effects have been reported in a wide range of applications in this regard [18,20,21,37], however no data have been announced concerning the toxicity of P/D scaffolds containing the CD-A molecule. The findings presented here support the non-toxicity of the interaction between the
polymeric system and the antioxidant molecule evaluated. Moreover, the foreign body reactions to biomaterials such as scaffolds are controlled by macrophage responses. Macrophages are heterogeneous cell populations, subtypes of which are M1 and M2. According to their functionality, M1 acts as a pro-inflammatory and tumorogenic macrophage, and M2 is regarded as the tissue repair macrophage \[38,39\]. CD68 antigen is expressed in both M1 and M2 populations, representing a classic sign of inflammation \[40\]; while CD163 is a specific phenotypic marker for M2 macrophage \[41\]. The histological results revealed a particularly important cellular reaction in the sham and antioxidant groups within the areas surrounding the scaffold. This reaction represents a classic response to implanted biomaterials, and was mainly arisen out of macrophages and giant cells, which play a central role in the tissue response to the presence of foreign bodies \[42–45\]. Additionally, a partial colocalization of CD163 surface marker with the zones also positive for CD68 in the 7 and 15 days of reperfusion, accompanied by a cellular positivity spread along the muscle area, could suggest an immunoregulation and constructive tissue remodeling process \[40,46,47\].

One of the most important cytoprotective systems against oxidative stress is the Keap1-Nrf2 complex \[48\]. Nrf2 is a pivotal transcription factor which regulates the expression of intracellular antioxidant genes, detoxifying enzymes, and several other ROS-neutralizing proteins \[49\]. Under physiological conditions, Nrf2 remains inactive in the Keap1-Nrf2 complex near the cell membrane. However, upon high ROS exposure, Nrf2 detaches from the Keap1-Nrf2 complex, translocates to the nucleus \[50\] and triggers the transcription of more than 200 genes including a set of cytoprotective enzymes, such as NQO1, glutathione peroxidase, HO-1, and superoxide dismutase \[51\]. It is widely expressed in oxygen-consuming organs, such as muscles, heart, blood vessels, liver, kidneys, and brain. Nrf2 translocation promotes cell survival, preserves cellular redox homeostasis, and plays a key role in reducing inflammation \[52\]. Through its enzymatic activity, NQO1 can prevent electron reduction of quinones, which results in the production of ROS. Indeed, it has been demonstrated that an increase in the NQO1 level is associated with a decrease of susceptibility to oxidative stress, while a mutation in the NQO1 gene increases the susceptibility to oxidative stress \[53\]. In this study, the immunohistological results suggest an indirect reduction of the oxidative stress response in the antioxidant-treated group, corroborating our previous findings where we showed the capacity of CD-A to indirectly activate the endogenous antioxidant system of human endothelial cells subjected to exogenous oxidative stress by the Nrf2/HO-1/NQO1 pathway. Moreover, our previous findings demonstrated an upregulation of HO-1 and NQO-1 protein expression levels when the cells received the CD-A treatment \[17\]. Similarly, a protective effect by astaxanthin pretreatment against brain injuries expressed by a significant increase of the Nrf2, HO-1, and NQO1 mRNA expressions in a cerebral ischemia model was also reported by Lei Pan et al. \[36\]. In a recent study, Shen et al. \[51\] showed the involvement of Nrf2 in myocardial I/R injury. The authors pointed out that during the acute myocardial infarction, Nrf2 combines with the antioxidant response element (ARE) to respond to the oxidative stress, reduce the cardiomyocyte apoptosis, and protect the normal function of myocardial tissue.

Iron is a crucial component in living organisms. It plays a vital role as a structural component of heme-containing proteins (Fe^{2+}) such as hemoglobin, myoglobin, and cytochromes, and as a metal cofactor (non-heme iron, Fe^{3+}) present in several enzymes including Fe-S cluster proteins in mitochondria. Iron homeostasis has been associated with oxidative stress and ROS generation \[54\]. One of the characteristic reactions of I/R is the reduction of non-heme Fe^{3+} and release of Fe^{2+} ions \[55\], which are able to react with H_{2}O_{2} to generate highly reactive hydroxyl radicals and result in cellular injury \[56,57\]. Owing to its high sensitivity, the Perls/DAB method was used to provide information about non-heme iron deposition regardless of oxidation states in normal and ischemic conditions by revealing cell population susceptible to free radical damage \[58,59\]. The results obtained here indicates a response to ROS generation, linked to the reperfusion period and to the non-heme ferrous iron production, which is considered to be critically involved in free radical generation.

Although we believe that an in situ delivery approach must be considered in the use of the astaxanthin molecule for the treatment of I/R-related pathologies like chronic arterial occlusive disease
of the lower extremities, we likewise recognize the limitations it imposes. The I/R model, evaluated here, is in accordance with the degree of ischemia seen in the patients [24]. However, by inducing a mild ischemia in a specific segment of the femoral artery, it seems essential to consider the irrigation of collateral arteries which could reduce the ischemic impact on the adjacent muscles such as the gracilis, thereby making it difficult to evidence the oxidative damage induced in the hind-limb and limiting the quantification of the possible treatment reached by means of the antioxidant. Moreover, as reported here, although in vitro results showed a high scavenging activity at 12.5 μM astaxanthin concentration, and in vivo results reflected a preliminary finding suggesting an improvement in the scavenging activity, by the in situ release of CD-A antioxidant molecule in the vicinity of the muscle in which oxidative stress was generated under I/R injury, an extensive evaluation is still required to select the most appropriate dose of astaxanthin in order to maximize the antioxidant effect.

In conclusion, the release of CD-A antioxidant through a P/D system in an I/R-damaged environment induces a positive immunological response reflected by an increase of the tissue repair macrophage markers and by the indirect modulation of the Nrf2 transcription factor and the expression of antioxidant enzymes susceptible to oxidative stress. Therefore, this study suggests that the local release of astaxanthin by a polysaccharide scaffold could contribute to the decrease of the damages in the muscles inflicted by I/R injury, by increasing the endogenous antioxidant defenses.

4. Materials and Methods

4.1. Chemical and Biological Reagents

Natural astaxanthin (purity >97% HPLC, powder, Lot: 5M4707V), Hydroxypropyl-β-cyclodextrin (DS = 0.67), potassium ferrocyanide (BioUltra, >99.5%), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), trisodium trimetaphosphate (STMP, lot# MKBQ7691V), sodium chloride (NaCl, lot# SZBA0490), and isopropanol (70% in H2O, Ref: 563935) were purchased from Sigma-Aldrich Co. LLC (Saint-Louis, MO, USA). Pullulan (MW 200000) was purchased from Hayashibara Inc., Okayama, Japan. Dextran (MW 500000), with a degree of branching of 5%, was obtained from Leuconostoc mesenteroides (Pharmacia, Uppsala, Sweden). Acetone, methanol, hydrochloric acid, hydrogen peroxide, and chloroform were all purchased form Carlo Erba Reagents S.A.S (Val-de-Reuil, France). EnVision Dako kit (ref K4063 and ref K4010), Dako REAL peroxidase-blocking solution, and Negative Control mouse IgG1 solution were provided by Dako (Dako, Carpinteria, CA, USA and Dako, Glostrup, Denmark). Mouse anti-rat CD68 and CD163 primary antibodies were obtained from Bio-Rad (MCA341A488, Marnes-la-Coquette, France). Anti-phospho-Nrf2 (S40) antibody was purchased from Abcam (ab76026, Paris, France). Mouse fibroblast cells CCL 163 (Balb/3T3 clone A31) were purchased from ATCC-LGC Standards S.a.r.l. (Molsheim Cedex, France). Minimum essential medium-L-glutamine (MEM), fetal calf serum (FBS), penicillin–streptomycin- amphotericin (PSA), and trypsin/EDTA solution (TE) were purchased from GIBCO (Life technologies, Carlsbad, CA, USA).

4.2. Pullulan/Dextran/CycloDextrin-Astaxanthin Scaffold Preparation and Loading

4.2.1. P/D Scaffold Preparation

Polysaccharide-based scaffolds were prepared according to the protocol proposed by Autissier et al. [60]. Briefly, pullulan, dextran, and NaCl (3:1:4) were mixed in 40% water (w/v). Then, 10 g of the polysaccharide solution was withdrawn, to which 1 mL of NaOH 10 M was added, and incubated at 50 °C for 20 min. Next, 300 mg of sodium trimetaphosphate (STMP) (30% w/v) in 1 mL of water was added as a chemical crosslinking agent. Finally, the mixture was poured into a glass mold of 10 cm × 5 cm × 1 mm. The resultant hydrogel was washed extensively in PBS pH 7.4, freeze-dried (Cryotec, Lyophilizer Crios, France), sterilized under UV light, and stored at room temperature until use.
4.2.2. CD-A Preparation

To facilitate natural astaxanthin loading into the scaffolds, hydroxypropyl-β-cyclodextrin was previously mixed with astaxanthin according to the following preparation method: Natural astaxanthin (A, 1 mg) in acetone/chloroform (v/v 1:1) solution was mixed with hydroxypropyl-β-cyclodextrin (CD, 250 mg) dissolved in 12.5 mL of 95% methanol and put in an atmosphere of nitrogen. The mixture was sonicated for 5 min at 35 °C (ultrasonic bath bandelin sonorex rx-100-h) and stirred overnight under light protection. The solution was dried under vacuum, recovered with double distilled water, freeze-dried, and stored at −4 °C until use.

4.2.3. Scaffold Loading and Characterization

Sterilized P/D scaffolds were cut in 5 × 5 mm squares and loaded with either 10 μL of saline solution for the sham group (P/D) or 2.5 mg CD-A reconstituted in 10 μL saline solution (12.5 μM) for the antioxidant group (P/D/CD-A). Five minutes were required for the solutions to be completely absorbed by the scaffolds. P/D and P/D/CD-A scaffolds were characterized by environmental scanning electron microscopy (ESEM) using a Philips XL 30 ESEM-FEG (Eindhoven, The Netherlands) at an accelerating voltage of 15 keV and at a pressure of 3.5 Torr.

4.2.4. Release Kinetics Evaluation

To determine the release of CD-A complex from the hydrogel, a calibration curve by plotting the CD-A absorbance (OD) at 472 nm against concentration of the complex (0–15 μM) was established (i-controlTM microplate reader software, TECAN Männedorf, Switzerland). The CD-A release from the scaffold complex was determined as follows: P/D/CD-A scaffolds were immersed in PBS 0.1 M, pH 7.4 at 37 °C, to allow the release of CD-A into the solution. Then, 50 μL of the release solutions were collected at 5, 10, 20, 30, 40, and 60 min. The absorbance of P/D/CD-A release products was recorded at 472 nm, and OD values were reported to the percentage of CD-A concentration based on the calibration curve.

4.2.5. CD-A Antioxidant Activity Evaluation

The antioxidant scavenging capacity of CD-A before and after release from the P/D scaffolds was evaluated using the ORAC (oxygen radical absorbance capacity) method [61]. Briefly, P/D scaffolds were previously loaded with CD-A (12.5 Mm) and immersed into the 1 mL PBS solution at 37 °C for 60 min.

Solutions of fluorescein (4 nM), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (160 μM, stressor), and Trolox (0–50 μM, antioxidant standard reference) were prepared in PBS. Fluorescein solution (150 μL) was added to each well (96-well microplate), and then 25 μL of the P/D/CD-A scaffold release product, blank (PBS), or Trolox were distributed in the wells, prior to the addition of AAPH (25 μL). Then fluorescence decay was monitored (485/ 528 nm Ex/Em) at 37 °C for 60 min, and data were taken every minute.

The area under the curve (AUC) of relative fluorescence was calculated. The AUC values for CD-A release products and Trolox were reported to the AUC value calculated for AAPH (stressor), to indicate the AUC net values. ORAC values were expressed as Trolox equivalent in μM (TEAC, μM) and calculated as the slope ratio from curves, AUCnet, versus the concentration of antioxidant and Trolox, respectively (AUCnet = AUCsample − AUCblank).

4.2.6. Cell Viability Assay

Cell viability was assessed using the MTT assay. To this end, mouse fibroblast cells (3T3) were grown in MEM, supplemented with 10% (v/v) FBS and 1% PSA. Then, the cells were seeded in a 96-well microplate at a cellular density of 1 × 10^4 cells/well and cultured overnight. After MEM removal, CD-A (1.25 to 5 μM) or P/D/CD-A scaffold release product, MEM (negative control, PC),
or 10% DMSO (positive control, NC) were added to the wells. After 24 h incubation in cell culture conditions, the solutions were discarded and the MTT assay was performed. In this regard, the MTT solution (200 μL, 0.5 mg/mL) was added to the cells and incubated for 3 h at 37 °C. Then, 200 μL of isopropanol was added for 20 min. Next, the absorbance was recorded at 570 nm (i-controlTM microplate reader software, TECAN Männedorf, Switzerland). The samples were considered non-toxic if cellular viability was higher than 70% compared to NC (based on the ISO 10993:2009 1-12 regarding the biological evaluation of medical devices).

4.2.7. Surgical Procedure and Experimental Design

Animal surgeries were performed in accordance with the “principles of laboratory animal care” and were approved by the Animal Ethics Committee of the Bichat Laboratory (N°2011-14/698-0038). Hind-limb I/R surgeries were performed on male rats weighted 250–300 g (Charles River Laboratory, Wilmington, MA, USA) using an I/R model adapted from Luyt et al. [62]. Animals were anaesthetized with intraperitoneal injection of pentobarbital (30 mg/kg) (Centravet, France). They were randomly assigned to three experimental groups: control (n = 12), sham (n = 12), and antioxidant (n = 12) (Figure 2). Control animals underwent 2 h of general anesthesia. The sham and antioxidant groups underwent 45 min of ischemia induced by the ligation of femoral artery with a 6-0 silk suture. Then an intramuscular incision, perpendicular to the muscle fibers, was made in the gracilis muscle, allowing the implantation and keeping the hold of the P/D scaffold charged with 10 μL saline solution in the sham group, or the P/D scaffold loaded with 12.5 μM of the antioxidant molecule (P/D/CD-A) in the antioxidant group, 5 min prior to the artery reperfusion. Then arterial blood flow was restored and reperfusion periods of 60 min, 7 or 15 days (Figure 2) were evaluated. Finally, rats were sacrificed with an overdose of sodium pentobarbital (150 mg/kg) intraperitoneally injected, and then the gracilis muscles were explanted and histologically evaluated.

4.2.8. Histological and Immunological Evaluation

Animals were sacrificed 1 h, 7 days or 15 days post-surgery. Gracilis muscles were collected and immersed in 4% formaldehyde during 24 h, dehydrated, and embedded in paraffin according to the standard protocols. Sections at 4 μm thickness (HM 3555 microtome, Thermo scientific Waltham, MA, USA) were stained with hematoxylin and eosin. Total phagocytes and type-2 macrophages were identified by anti-CD68 and anti-CD163 antibodies labeling respectively (1:30 working dilutions). Anti-phospho Nrf2 (1:100 working dilution) immunomarker was used to collect information about the muscle general oxidative stress status after I/R injury. DAKO EnVision+ System and 3,3’-diaminobenzidine was used as a chromogen. Iron accumulation in healthy and I/R muscles was quantified by Perls reaction amplified by 3,3’-diaminobenzidine (DAB). After antigen retrieval, samples were incubated in a solution (pH 0.5–0.6, 25 °C) containing 1% HCl and 1% potassium ferrocyanide, followed by H2O2 incubation [63]. All slides were counterstained with hematoxylin, and digital images were obtained and analyzed using Nanozoomer digital pathology software (Hamamatsu, Japan).

Samples images were analyzed by two independent methods:

(i) A mathematical quantitative program: A saturation analysis which highlighted the positive stains in the images created by different immunostaining markers using Matlab software (version 8.5, The MathWorks, Inc., Natick, MA, USA). Each image was separated into its components: hue, saturation, and value color bands. Then thresholds were defined to determine the mask for the region of color corresponding to the positively stained cells represented by brown color spots. Total image pixels of each image were used to calculate the percentage of positive staining as a pixel difference.

(ii) A qualitative method: The analysis of the proximal and distal sections of the muscle was performed by a physician/pathologist in an independent and random manner. The tissue response was scored (macrophages, cellular infiltration, giant cells) according to a relative scoring system [64]: – = no observation, + = mild, and ++ = moderate.
4.2.9. Statistical Analysis

All experiments were repeated at least three times to ensure the reproducibility of each test. Results were expressed as the mean ± SD and statistical analyses were done using one-way ANOVA followed by Tukey’s HSD (honestly significant difference) post hoc test (JMP Software, Version 9; SAS Institute, Cary, NC, USA). Statistical significance was set at p-value < 0.05.

4.2.10. List of abbreviations

Reactive oxygen species (ROS); ischemia/reperfusion (I/R); hydroxypropyl-β-cyclodextrin (CD); hydroxypropyl-β-cyclodextrin-astaxanthin (CD-A); pullulan/dextran-based scaffold (P/D); pullulan/dextran-based scaffold loaded with hydroxypropyl-β-cyclodextrin-astaxanthin (P/D/CD-A scaffold); oxygen radical absorbance capacity (ORAC); area under the curve (AUC); nuclear factor-erythroid 2-related factor 2 (Nrf2); heme oxygenase-1 (HO1); NAD (P) H: quinone oxidoreductase 1 (NQO1).

Supplementary Materials: The following are available online at http://www.mdpi.com/1660-3397/17/6/354/s1,
Figure S1: Calibration curves of CDA and ORAC test, Figure S2: Hematoxylin-eosin staining of liver and kidney from control rats and rats which underwent 45 min of ischemia and were subjected to 7 or 15 days of perfusion, Figure S3: Immuno-histological staining of rat and mouse heart showing the positivity of the antibody anti-phospho-Nrf2, Figure S4: Quantification of positive staining using Matlab.

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Abstract. Increased plasma levels of homocysteine (Hcy) can cause severe damage to vascular endothelial cells. Hcy-induced endothelial cell dysfunction contributes to the occurrence and development of human cerebrovascular diseases (CVDs). Our previous studies have revealed that astaxanthin (ATX) exhibits novel cardioprotective activity against Hcy-induced cardiotoxicity in vitro and in vivo. However, the protective effect and mechanism of ATX against Hcy-induced endothelial cell dysfunction requires further investigation. In the present study, treatment of human umbilical vascular endothelial cells (HUVECs) with Hcy inhibited the migration, invasive and tube formation potentials of these cells in a dose-dependent manner. Hcy treatment further induced a time-dependent increase in the production of reactive oxygen species (ROS), and down-regulated the expression of vascular endothelial growth factor (VEGF), phosphorylated (p)-Tyr-VEGF receptor 2 (VEGFR2) and p-Tyr397-focal adhesion kinase (FAK). On the contrary, ATX pre-treatment significantly inhibited Hcy-induced cytotoxicity and increased HUVEC migration, invasion and tube formation following Hcy treatment. The mechanism of action may involve the effective inhibition of Hcy-induced ROS generation and the recovery of FAK phosphorylation. Collectively, our findings suggested that ATX could inhibit Hcy-induced endothelial dysfunction by suppressing Hcy-induced activation of the VEGF-VEGFR2-FAK signaling axis, which indicates the novel therapeutic potential of ATX in treating Hcy-mediated CVD.

Introduction

Endothelial dysfunction has been identified as one of the most important pathogenetic causes of human cerebrovascular disease (CVD) (1,2). Endothelial dysfunction can cause damage to the blood-brain barrier and can result in a range of neurological disorders, including multiple sclerosis, vascular dementia and subsequent complications of the extremities (3-5). Cerebral small vessel disease is a condition that involves the formation of white matter lesions and cerebral microbleeds, and has been associated with endothelial dysfunction (6).

Elevated serum levels of homocysteine (Hcy) is an independent risk factor that can damage vascular endothelial cells and can cause endothelial dysfunction, which in turn contributes to the occurrence and development of CVDs (7-9). Several studies have focused on the ability of Hcy to lower the severity of numerous human diseases (10-12). Hcy-induced apoptosis of endothelial cells has been reported to account for Hcy-dependent vascular injury (13). Accumulated evidence suggests that Hcy can cause endothelial dysfunction. For example, Hcy can inhibit endothelial nitric oxide (NO) synthase signaling (14) and cell migration by targeting key angiogenic factors (15). Furthermore, it can reduce the expression levels of vascular endothelial growth factor (VEGF)-A and vascular endothelial growth factor receptor (VEGFR)-2 (16,17). Hcy can inhibit microvascular endothelial cell formation by disrupting cell migration via an inducible NO synthase-dependent mechanism (18,19). Hcy can decrease the invasive potential of endothelial cells by inhibiting matrix metalloproteinase (MMP)-2 and urokinase (19); however, the mechanism of cytotoxicity of Hcy on endothelial cells remains unclear. Furthermore, to the best of our knowledge, the role of reactive oxygen species (ROS) in endothelial dysfunction has not been investigated previously.
Astaxanthin (ATX) is a potent antioxidant that undertakes a novel mechanism of action. Our previous study revealed that ATX can attenuate Hcy-induced cardiotoxicity in vitro and in vivo by inhibiting mitochondrial dysfunction and oxidative damage (20). It was reported that ATX could attenuate the astrocyte apoptosis and reduce traumatic brain injury by inhibiting Na-K-Cl co-transporter (NKCC1) and the secretion of proinflammatory cytokines (21). These effects were caused by the suppression of oxidative stress and the upregulation of brain-derived neurotrophic factor and nerve growth factor mRNA (22,23). ATX exerted neuroprotective effects against subarachnoid hemorrhage damage that involved the inhibition of MMP-9 expression, the upregulation of Akt/glycogen synthase kinase-3β and the activation of the nuclear factor-like 2-antioxidant responsive element pathway (24-32); however, the protective effects of ATX against Hcy-induced endothelial dysfunction and the underlying mechanism require further investigation.

**Materials and methods**

**Materials.** Dulbecco's Modified Eagles medium (DMEM)/F-12 and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc.). ATX (purity, 97%), Hcy (purity, 98%), MTT and propidium iodide were obtained from Sigma-Aldrich (Merck KGaA). All primary antibodies used in the present study, including anti-VEGF (cat. no. 2463), VEGFR2 (cat. no. 9698), phosphorylated (p)-VEGFR2 (cat. no. 2478), Tyr937-focal adhesion kinase (FAK; cat. no. 3283), FAK (cat. no. 3285) and β-actin (cat. no. 8457) were purchased from Cell Signaling Technology, Inc. A horseradish peroxidase-linked goat anti-rabbit immunoglobulin G (cat. no. 7074; Cell Signaling Technology, Inc.) was used as the secondary antibody. PF-562271 was purchased from Selleck Chemicals. All solvents used were of high-performance liquid chromatography grade.

**Cell viability assay.** Human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection. HUVECs were cultured in DMEM-F12 containing 10% FBS at 5% CO₂ and 37°C in an incubator.

**Cell migration assay.** HUVECs were seeded in a 6-well tissue culture plate and cultured at 37°C for 24 h. Scratched wounds were created by scraping the cell monolayer with a sterile 10 µl pipette tip. Subsequently, the cells were cultured with DMEM/F-12 medium (containing 1% FBS). Subsequently, the cells were pre-treated with 5 µM ATX for 6 h and/or 10 mM Hcy or 10 nM PF562271 at 37°C for 48 h. Untreated cells were used as control. The migrated cells were imaged in five randomly-selected fields of view with a phase-contrast microscope (magnification, x200) and the percentage of migration was quantified by manual counting (% of control).

**Cell invasion assay.** HUVECs were pre-treated with 5 µM ATX for 6 h and/or co-incubated with 10 mM Hcy at 37°C for 72 h. Following treatment, HUVECs (4x10⁴ cells/well) were suspended in 100 µl DMEM/F-12 medium (FBS-free) and were seeded in the upper layer of a Matrigel pre-coated Transwell chamber. Complete DMEM/F12 (600 µl, 10% FBS) was added into the lower chamber. Following a 24 h incubation period at 37°C, the non-invaded cells on the Transwell were removed using a cotton swab; invaded cells were washed with PBS, fixed with 10% ethanol for 10 min at room temperature (25°C) and stained with 0.1% crystal violet for 15 min at room temperature (25°C). Invaded cells were measured by manual counting with a Nikon Ti-S inverted microscope (magnification, x100). In total, five randomly-selected fields of view per sample were imaged and analyzed.

**Tube formation.** In vitro tube formation was examined by a Transwell assay. Briefly, HUVECs were pre-treated with 5 µM ATX for 6 h and/or co-incubated with 10 mM Hcy at 37°C for 72 h. Following treatment, HUVECs (10⁴ cells/well) were seeded in Matrigel pre-coated 48-well plates and incubated at 37°C for 24 h. In total, five randomly-selected fields of view per sample were imaged, and the number of tubes formed manually counted using a Nikon inverted microscope (magnification, x100).

**ROS measurement.** The levels of intracellular ROS in HUVECs were detected by the 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, HUVECs were incubated with 10 µM DCFH-DA for 20 min at 37°C in the dark. Subsequently, the cells were washed with PBS and treated with 10 mM Hcy at 37°C for 10, 30, 60 and 120 min. On the contrary, cells were treated with 5 µM ATX for 60 min and/or co-treated with 10 mM Hcy at 37°C for 120 min to analyze the protective effects of ATX. For ROS inhibition, cells were pre-treated with 5 mM glutathione (GSH) at 37°C for 2 h prior to ATX/Hcy treatment. The production of ROS was quantified using a microplate reader by measuring the fluorescence intensity at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

**Western blotting.** Protein expression was detected by western blotting. Briefly, HUVECs were pre-treated with 5 µM of ATX for 6 h and/or co-incubated with 10 mM Hcy at 37°C for 72 h. Following treatment, the cells were collected and lysed on ice for 1 h at 4°C in RIPA lysis buffer (Nanjing KeyGen Biotech Co., Ltd.). Total protein was quantified with a Bicinchoninic Acid detection kit. A total of 40 µg of protein was added and separated on a 10% SDS gel at 110 V for 75 min. Following electrophoresis, the proteins were transferred from the gel onto the nitrocellulose membrane. The membrane was blocked.
with 5% non-fat milk at room temperature (25°C) for 1 h and incubated overnight with a primary antibody (1:1,000) for 1 h at room temperature (25°C). The target protein was scanned with X-ray film using an enhanced chemiluminescence system (Kodak). β-actin was used as the reference protein.

**Statistical analysis.** The experiments were conducted with the SPSS software (version 13.0; SPSS, Inc.). Data are presented as the mean ± SD. Statistical evaluation was analyzed by one-way ANOVA followed by a Dunnett’s or Tukey’s post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**ATX inhibits Hcy-induced cytotoxicity in HUVECs.** Initially, the toxicity of Hcy towards HUVECs was examined by an MTT assay. Hcy alone apparently suppressed HUVEC viability in a dose-dependent manner (Fig. 1A). Treatment of HUVECs with 5, 10 and 20 mM Hcy significantly suppressed the cell viability from 100% (control) to 77.1, 56.2 and 25.5%, respectively (Fig. 1B). ATX (10 µM) alone indicated no cytotoxicity towards HUVECs. In addition, ATX pre-treatment ameliorated morphological changes induced by Hcy in HUVECs. Hcy treatment notably decreased cell number, and induced cell shrinkage (Fig. 1C). These results suggested that ATX could inhibit Hcy-induced cytotoxicity in HUVECs.

**ATX increases cell migration, invasion and tube formation in Hcy-treated HUVECs.** To examine the effects on the functions of endothelial cells, we examined HUVEC migration, invasion and tube formation, which are considered indices of angiogenesis. Initially, Hcy-treated HUVEC migration was analyzed by a wound-healing assay. Hcy treatment alone (10 mM) significantly inhibited the migration rate from 100% (control) to 17.9%; however, ATX pre-treatment (5 µM) significantly improved the migration rate to 77.8% (Fig. 2B). ATX treatment alone indicated no significant effect on HUVEC migration compared with untreated cells. The potency of ATX was further examined using cell invasion and tube formation assays. Hcy treatment alone (10 mM) significantly inhibited cell invasion and tube formation compared with the untreated control, whereas ATX pre-treatment (5 µM) significantly improved cell invasion and tube formation in Hcy-treated cells.

Figure 1. ATX inhibits Hcy-induced cytotoxicity in HUVECs. (A) Cytotoxicity of Hcy towards HUVECs. Cells (8,000 cells/well) were seeded in 96-well plate and treated with Hcy for 72 h. (B) ATX pre-treatment inhibited Hcy-induced HUVEC cytotoxicity. Cells were pretreated with 1-10 µM ATX for 6 h and co-treated with 10 mM Hcy for 72 h. Cell viability was detected by an MTT assay. (C) Morphological changes of HUVECs. Following treatment, cells were observed under a phase-contrast microscope (magnification, x400). All data and images were obtained from three independent experiments. *P<0.05, **P<0.01 vs. control; #P<0.05, ##P<0.01 vs. Hcy-treated group. ATX, astaxanthin; Hcy, homocysteine; HUVECs, human umbilical vascular endothelial cells.
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Collectively, these results indicated that ATX could improve cell migration, invasion and tube formation in Hcy-treated HUVECs.

ATX inhibits Hcy-induced effects on the VEGF-VEGFR2-FAK signaling pathway. Accumulating evidence has suggested that the VEGF-VEGFR2-FAK is one of the most important pro-angiogenic signaling pathways that serve a key role in regulating cell migration, invasion and tube formation (33). This pathway can be potentially targeted for therapeutic intervention. Therefore, in the present study, the expression levels of proteins involved in the VEGF-VEGFR2-FAK pathway were detected by western blotting. Treatment of cells with Hcy induced a significant time-dependent decrease in the expression of VEGF, p-Tyr-VEGFR2 and p-Tyr397-FAK (Fig. 3A). Notable changes were noted in the expression levels of total-FAK and total-VEGFR2 in Hcy-treated cells. To further evaluate the role of FAK, we used the FAK inhibitor, PF562271. The results indicated that treatment with PF562271 markedly enhanced the Hcy-induced inhibition of p-Tyr397-FAK expression (Fig. 3B). Additionally, PF562271 and Hcy significantly inhibited of HUVEC migration compared with the control (Fig. 3D), which suggested that Hcy inhibited HUVEC migration in a FAK-dependent manner. However, ATX pre-treatment markedly recovered the expression of p-Tyr397-FAK in HUVECs that were induced by the combined treatment of the FAK inhibitor and Hcy (PF562271 + Hcy). ATX pre-treatment (5 µM) reversed the effects of combined treatment of PF562271 and Hcy on FAK phosphorylation (Fig. 3C). In addition, ATX pre-treatment significantly increased the rate of migration of HUVECs (47.8%) compared with the combined treatment (8.99%; Fig. 3E). Collectively, these findings indicated that ATX could inhibit Hcy-induced dysfunction of the VEGF-VEGFR2-FAK signaling pathway.

ATX inhibits ROS-dependent FAK phosphorylation. Accumulating evidence has shown that Hcy can induce ROS accumulation, which can further cause cytotoxicity (20-22). Therefore, the intracellular accumulation of ROS in Hcy-treated HUVECs was examined. Hcy treatment induced...
ROS production in a time-dependent manner, as demonstrated by the enhanced green fluorescence (Fig. 4A); however, ATX pre-treatment effectively inhibited Hcy-induced ROS production (Fig. 4B). In addition, ATX recovered the levels of Tyr397-FaK phosphorylation and improved HUVEC viability (Fig. 4C-E), which indicated similar protective effects to those of GSH, a ROS scavenger. The results suggested that Hcy induced ROS-dependent FaK phosphorylation; inhibition of ROS formation by ATX or GSH may increase FaK phosphorylation. Collectively, these results suggested that ATX could inhibit ROS-dependent FaK phosphorylation in Hcy-treated HUVECs.

**Discussion**

Numerous studies have supported the notion that hyperhomocysteinemia can induce endothelial cell apoptosis and promote the development of vascular diseases (10-17). This condition has therefore emerged as an independent risk factor for human CVD (34). The pathogenesis of hyperhomocysteinemia-associated human CVD is remains unclear, but may be due to dysregulated endothelial cell migration and invasion. Angiogenesis is a critical process required for physiological processes in the body, such as the regeneration of the damaged vascular tissues. The process of angiogenesis includes capillary or posterior venous endothelial cell activation, proliferation and migration. In addition, endothelial cell migration is one of the most important processes of angiogenesis. Endothelial cells can invade surrounding tissues, a prerequisite for the development of angiogenesis in response to migration signaling (2,3). Hyperhomocysteinemia may cause damage to vascular endothelial cells and consequently inhibit cell migration. The morphology of viable cells following Hcy treatment was notably altered than that of the control group, as determined by phase-contrast microscopy. These findings indicated that Hcy affected the normal function of endothelial cells. Atherosclerosis and cerebral hemorrhages are complex processes initiated at sites of endothelial cell injury. Injured endothelial cells can cause the endothelium-dependent relaxation of blood vessels, thereby resulting in the development of CVDs (35). In the present study, Hcy treatment significantly inhibited the migration and invasive potentials of HUVECs.
compared with the control group. Thus, inhibiting endothelial cell migration and invasion may suppress the process of angiogenesis.

The formation of a mature vascular network is inhibited with vessel destabilization, followed by endothelial cell re-organization. This process is completed by vessel maturation (10-17). Angiogenesis requires the simultaneous precise regulation of a large number of angiogenic factors, including VEGF and VEGFR2, and their downstream signaling proteins, namely ERK, AKT and FAK (36). The VEGF-VEGFR2 axis aids endothelial cell recruitment and vascular permeability, whereas ERK activates endothelial cell proliferation; FAK promotes cell migration and invasion. VEGF and VEGFR2 have been considered to be the most important factors in this pathway, and serve key roles in regulating angiogenesis via the modulation of the degradation, differentiation, proliferation and migration of vascular endothelial cells (36-40). The VEGF-VEGFR axis eventually promotes the formation of new blood vessels (36-39). In clinical settings, patients with hyperhomocysteinemia usually possess endothelial cells with impaired endothelial activities, including cellular proliferation, migration and adhesion, which can harm human heart health (41-43). The present study revealed that Hcy induced endothelial cell dysfunction, and these effects were reversed by ATX pre-pretreatment, possibly via the regulation of FAK activation and increased cell migration in Hcy-treated HUVECs. Our findings provide insight into the potential therapeutic role of ATX in the prevention and chemotherapy of Hcy-mediated human CVDs.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JKM designed the experiments. XJW, DCT, FWW, XYF and CDF performed the experiments. MHW and XYF analyzed the data and prepared the images. JKM and XJW wrote the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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PROTECTIVE EFFECT OF ASTAXANTHIN IN THE LUNG INJURY CAUSED BY ISCHEMIA REPERFUSION OF THE LOWER EXTREMITIES

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ABSTRACT

Pathological and biochemical alterations due to lower extremity (I/R) damage and protective effects of astaxanthine (AST) were investigated. Rats were divided into four groups. GI-Sham group (n=7): Anesthesia without (I/R)(2hours); GII-I/R (n=7): 2 hours of ischemia and 2 hours of reperfusion under anesthesia; Group III-AST(n=7): Rats were subchronically orally administered for 7 days at 125 mg/kg astaxanthin (AST) and then anesthetized (2hours) without ischemia; GIV-I/R+AST (n=7): 7 days prior to ischemia rats were subchronically orally administered 125 mg/kg astaxanthin (AST) and then 2 hours of ischemia and reperfusion under anesthesia; Then lung tissues were investigated for MDA, GSH and histopathology. An increase in MDA and a decrease in GSH was observed I/R administered group compared to control. Histopathological evaluations showed intense congestion in pulmonary veins and alveolar septum and partial alveolar macrophage and erythrocyte accumulation and edema was observed in lumens of some bronchioles and alveoli in the second and fourth group compared control. Second group (3.41) damage score had high significance compared to control (p<0.001). Fourth group damage score (0.92) was indifferent from control but significantly different from I/R group (p<0.001). As a result, The protective effect of AST has been demonstrated by biochemical, histopathological and immunohistochemical effects.

Keywords: Lung, Astaxanthin, Rat, MDA, GSH, Histopathology.

INTRODUCTION

Lower extremity Ischemia/reperfusion (I/R) damage is a frequently encountered phenomenon in surgical interventions (Grace, P.A. 1994). Ischemia and following reperfusion triggers local and systemic damage with the involvement of free oxygen radicals and inflammatory mediators (Tekeli et al. 2001; Uysal et al. 2006). Although blood flow saves extremity from necrosis, multi organ dysfunction may progress and cause death of the patient. In general, this damage is observed in heart, kidney and lung tissue (Blaisdell et al. 2002; Bengisu et al. 1997). Following lower extremity I/R damage, lungs are targets in end-organ damage and this damage can cause very important clinical aspects. Although etiology is not clarified yet, some humoral mediators in reperfusion are blamed for this end organ damage (Fantini et al. 1995; İspir et al. 2000). Astaxanthin (AST; 3,30-dihidroksi-β, β-karoten-4,40-dion), is found in various organisms and especially in salmon, sea bream, rainbow trout, shrimp, lobster, fish eggs and algae. This lipid soluble xanthophil has strong antioxidant functions. AST scavenges single oxygen atoms as well as other free radicals and avoids or terminates peroxidation chain reactions (Gal et al. 2012; Nakagawa et al. 1997; Oshima et al. 1993). AST, is a known as a prefect antiinflammatory agent since it suppresses proinflammatory cytokine and chemokine expression (Yeh et al. 2016). AST, is used in treatment of cardiovascular disease (Pashkow et al. 2008), ischemic brain injury (Shen et al. 2009), cataract (Wu et al. 2006), diabetes (Uchiyama et al. 2002), hepatocellular injury due to ischemia (Curek et al. 2010) and diabetic nephropathy (Manabe et al. 2008; Naito et al. 2004).

Aim of this study is to evaluate protective effect of AST in the lung injury caused by ischemia reperfusion of the lower extremities.

MATERIALS AND METHODS

Animals: Study was conducted at Bingöl University Animal Experiments Center after approval of Bingöl University Animal Experiments Local Ethical Committee (18.11.2015 -2015/08). Twenty-eight male Wistar-albino rats weighing 250-300 grams were used at 7-8 weeks of age. Animals were kept in cages in controlled rooms with 20-22°C constant temperature and 12 hour cycles of light-dark (lights were on between 07:00-19:00; dark between 19:00-07:00). Water and standard food were given ad libitum. Rats were let for adaptation for one week in their
cages and experiments started after adaptation period. Feeding was ceased 12 hours before experiments whereas water was provided throughout the experimental period.

**Experimental Design:** Commercially obtained AST was subchronically administered to Sprague-Dawley rats orally. Rats were divided into four groups each containing 7 animals; Group I (Sham Group, n=7): Rats in this group was administered with anesthesia (2 hours) without ischemia-reperfusion and tissue samples were obtained. Group II (Ischemia/Reperfusion (I/R, n=7): Rats were administered with 2 hours of ischemia and following 2 hours of reperfusion under anesthesia (Bilgiç et al. 2018), Group III (AST, n=7): Rats in this group was administered with subchronic orally AST for 7 days at 125 mg/kg dosage and following anesthesia for 2 hours without ischemia (Gross et al. 2006). Group IV (Ischemia/Reperfusion (I/R) + AST (n=7): Rats in this group was administered with subchronic orally AST for 7 days at 125 mg/kg dosage, then administered with two hours of ischemia and two hours of reperfusion under anesthesia then lung tissue samples were obtained. Rats were anesthetized with 60 mg/kg i.p. ketamine hydrochloride and 10 mg/kg Xylazine i.p. Lung tissue was removed after median laparotomy, washed with PBS, stored in deep freeze (-80°C) and biochemical (MDA,GSH) and histopathology were performed.

**Preparation of tissue extract:** Tissue samples were washed with isotonic solution, dried and weighed. They were kept at -80 °C for analyses. Samples taken out from freezer were placed into tubes and Tris-buffer was added 10 times of their weight. They were disintegrated with homogenizer and placed into +4 °C freezer. Following vortex, cell membrane was lysed with ultrasonic bath in 10 s intervals for 30 s. They were centrifuged at 16000 rpm for 30 min. Supernatants were transferred into Eppendorf tubes. MDA and GSH levels were determined with such supernatants of lung tissue.

**BIOCHEMICAL ASSAYS**

**Measurement of MDA Level:** 50 mg tissue sample was homogenized with 0.15 mol/L KCl solution. Homogenates were centrifuged at 1600× g. MDA levels in tissues were determined with thiobarbituric acid (TBA) reaction according to Placer et al. (Placer et al. 1966).

**Measurement of GSH Level:** Tissue GSH levels were measured at 412 nm according to Sedlak and Lindsay (1968). Samples were precipitated with 50 % TCA and centrifuged at 1000× g for 5 minutes. Reaction mixture contained 0.5 mL supernatant, 2.0 mL EDTA (0.2 M, pH: 8.9) buffer and 0.1 mL 0.01 M DTNB. Solution was kept in room temperature for 5 minutes and then read at 412 nm with spectrophotometer.

**Total protein concentration determination:** The total protein concentration in the lung tissue homogenate was according to Lowry (1951). Total protein concentration in lung tissue homogenate was conducted by using bovine serum albumin as standard. A standard graphic was set by using standard bovine serum albumin standard solution. Measurements were performed at 695 nm wavelength.

**Histopathological and immunohistochemical investigations:** At the end of experimental procedure all rats were necropsied and lung tissues were taken. Lung tissue samples for histopathological investigation were fixed with 10 % buffered formalin solution for 48 hours. Tissue samples were passed from alcohol series for dehydration and from xylol series for clarification then blocked in paraffin. From those blocks serial cross sections were obtained with 4 micrometers thickness by using a microtome (Leica RM 2135). Samples were stained with Hematoxyline-Eozin (H.E.) and investigated and photographed under light microscope (Nikon 80i-DS-R2). Histopathological findings were partially modified and evaluated semi-quantitatively. Such criteria were; alveolar generation, thickening in interalveolar septum, capillary hyperemia, hemorrhage, inflammatory cell infiltration, terminal bronchiole structure, terminal bronchiole debris, respiratory bronchiole wall structure, respiratory wall debris, alveolar structure and edema in vascular wall. Its grading were done accordingly; 0=no alteration; 1=focal light alterations; 2=multifocal light alteration; 3=multifocal apparent alteration; 4=diffuse light alteration; 5=diffuse apparent alteration (Uysal et al. 2006). For immunohistochemical investigation slices of 4 μm thickness were placed in between poly-lysinecoated slides. To show eNOS immuno reactivity, anti-eNOS primary antibodies were used and stained with Avidin-Biotin Peroxidase Complex (ABC) immunohistochemical method. Obtained slices were incubated for 2 hours at 60°C and administered with two different xylol for clarification and rehydrated with decreasing alcohol series. Finally they were remained in distilled water for 5 minutes. Slices were kept in room temperature for 20 minutes in 0.5% trypsin solution. In addition they were kept in 3% hydrogen peroxide for 5 minutes to avoid endogenous peroxidase. Slices were washed with PBS for 5 minutes 3 times and incubated at +4°C overnight after eNOS primary antibody administration. Following day, slices were washed 3 times with PBS and let for 30 min incubation with biotinylated secondary antibody. They were bound with secondary antibody Vector Elite ABC kit. They became visible by dropping diaminobenzidine (DAB chromagen) which is the coloration substance for antibody-biotin-avidin-peroxida secomplex. Background staining was done with Mayer’s hematoxylene. Dehydration was performed with graded alcohol and clarification with xylol. Finally they were covered with entellan. Immunohistochemical staining intensity was evaluated by a double blind pathologist. eNOS staining
RESULTS

Biochemical findings: MDA level was increased in I/R administered group compared to control as expected. MDA level was lower in I/R+AST administered group compared to I/R group significantly (p<0.05). MDA level in AST group was found statistically lower compared to I/R group (p<0.01) (Figure1). GSH was decreased in I/R group compared to control(p<0.05). On the other hand in AST administered group, GSH value was increased compared to I/R group. This increase was found statistically significant (p<0.05)(Figure2).

Histopathological and immunohistochemical findings: No macroscopic alteration in lungs of rats was observed in groups. Microscopic evaluation of control group (Figure 3A) and AST administered group (Figure 3F) revealed no histopathological finding and lung parenchymatic tissue was in normal appearance. In histopathological investigation of rats in I/R group some intense histopathological findings were found. Intense congestion in pulmonary veins and inter alveolar septum was observed. In addition, there were macrophage and erythrocyte accumulation and edema in some parts of bronchioles and alveolar lumens. Hyperemia, bleeding and inflammatory cell infiltration in capillaries in inter alveolar septums were observed. Those findings showed an interstitial pneumonia with enlarged alveolar septums. In some cases perivascular and peribronchiolar mononuclear cell infiltrations were also encountered. Partial bronchiolar and alveolar epithelial cell degeneration and necrosis as well as desquamation were also observed. In addition, there was muscular hyperplasia in smooth muscle layers of some vessels and bronchioles. Due to intense exudate accumulation, congestive atelectasis with different intensities, enlargement of some alveoli in emphysematous fashion and even destruction of morphological structure due to rupture of alveolar walls were observed (Figure 3B-3D). In group administered with AST and I/R, hyperemia, hemorrhage, edema, inflammatory cell infiltration and partial enlargement in inter alveolar septum were observed (Figure 3E). Such findings are quite light compared to I/R group and resembles control group in general appearance. In semi quantitative histopathological evaluation, lungs revealed no damage at 4th or 5th grade. Lowest calculated score was for control (0.69) and AST groups (0.72) without statistical difference in between. I/R group showed the highest damage score with 3.41. Difference of this group with control group gives high statistical significance (P≤0.001). Damage score of concomitant AST and I/R administered group was 0.92. This value was insignificant compared to control but showed statistically significant difference with I/R group (P≤0.001). Immunohistochemical evaluation of vessel endothelium showed no eNOS positive staining (-) in control group and AST administered group. In general I/R group rats showed strong eNOS staining (+++) in lung vessel endothelium (Figure 3G) whereas I/R+AST group had low eNOS staining (+) (Figure 3H).

Figure 1. MDA levels of groups (b=p≤0.01,c=p≤0.05).

Figure 2. GSH levels of groups (c,c1=p≤0.05)
Figure 3. A) Control Group; normal histological architecture of lung, HandE X 4. B) I/R Group; intense hemorrhage (stars), MNL infiltration in peribronchioles and interalveolar septum ( ), exudate in bronchial lumen (a), emphysema in alveoli ( ), HandE X10. C) I/R Group; peribronchiolar and perivascular MNL infiltration ('), intense hemorrhage (stars) and muscular hyperplasia in bronchioles epithelial level (arrow heads), HandE X10. D) I/R Group; hemorrhage and thickening in interalveolar septum, MNL infiltration ('), emphysema ( ) and atelectasis (arrows), HandE X10. E) I/R + AST Group; nearly normal appearance in alveoli except very weak hemorrhage and edema in some interstitial area (stars), HandE X10. F) AST group; normal histological architecture of lung, HandE X10. G) I/R Group; intense eNOS positive staining in vessel endothelial cells (arrows). Immunoperoxidase X20. H) I/R + AST Group; low eNOS positive staining in vessel endothelial cells (arrows). Immunoperoxidase X20.
Table 1. Histopathological findings of protective effect of AST on lung tissue in experimental I/R in rats.

| Parameters                                | Control       | I/R          | I/R + AST     | AST          |
|-------------------------------------------|---------------|--------------|---------------|--------------|
| Perivascular and peribronchiolar inflammatory cell infiltration | Low: 2/8<sup>b</sup> | 8/8<sup>a</sup> | 3/8<sup>b</sup> | 2/8<sup>b</sup> |
|                                           | Moderate: - 2 | 1            |               |              |
|                                           | Intense: - 6  | -            | -             |              |
| Congestion and capillary hyperemia        | Low: 2/8<sup>b</sup> | 8/8<sup>a</sup> | 3/8<sup>b</sup> | 2/8<sup>b</sup> |
|                                           | Moderate: - 3  | 3            | 1             | -            |
|                                           | Intense: - 5  | -            | -             | -            |
| Hemorrhage in interstitial                | Low: -/8<sup>b</sup> | 8/8<sup>a</sup> | 3/8<sup>b</sup> | -/8<sup>b</sup> |
|                                           | Moderate: - 2  | 1            | -             | -            |
|                                           | Intense: - 6  | -            | -             | -            |
| Edema                                     | Low: -/8<sup>b</sup> | 8/8<sup>a</sup> | 3/8<sup>b</sup> | -/8<sup>b</sup> |
|                                           | Moderate: - 3  | 2            | -             | -            |
|                                           | Intense: - 4  | -            | -             | -            |
| Thickening in interalveolar septum        | Low: -/8     | 8/8<sup>*</sup> | 4/8<sup>*</sup> | 2/8         |
|                                           | Moderate: - 1  | 4            | -             | 2            |
|                                           | Intense: - 7  | -            | -             | -            |
| Emphysema in alveoli                      | Low: -/8     | 8/8<sup>*</sup> | 4/8<sup>*</sup> | -8          |
|                                           | Moderate: - 3  | 2            | -             | -            |
|                                           | Intense: - 5  | -            | -             | -            |
| Atelectasis in alveoli                    | Low: -/8     | 8/8<sup>*</sup> | 4/8<sup>*</sup> | -8          |
|                                           | Moderate: - 3  | 2            | -             | -            |
|                                           | Intense: - 5  | -            | -             | -            |
| eNOS immunoreactivity                     | Low: -/8     | 8/8<sup>*</sup> | 6/8<sup>*</sup> | -8          |
|                                           | Moderate: - 1  | 1            | -             | -            |
|                                           | Intense: - 7  | -            | -             | -            |

**DISCUSSION**

I/R damage is due to free radical formation following re-oxygenation of the tissue after reperfusion of the tissue (Prem et al. 1999). Reactive oxygen damage may arise from more than one source and among them the most important ones are the activated neutrophils (Cetinkale et al. 1998; Pararajasingam et al. 1999; Cavanagh et al. 1998). Role of neutrophils in I/R damage is pronounced (Isip et al. 2000; Cetinkale et al. 1998; Lee et al. 1992). Most important factor for lung damage is via formation of free oxygen radicals and proteolytic enzymes released by neutrophils attached to endothelium (Berkan et al. 2001). Increased PMNL activity which is formed by I/R damage causes chemio attraction and infiltration of PMNL which is followed by their degranulation. After degranulation, free radical formation increases and proteases cause lung endothelium damage and augmented pulmonary capillary permeability (Nelson et al. 2001; Seekamp et al. 1993; Cohen et al. 1997). Recently use of substances such as superoxide dismutase, allopurinol, catalase, mannitol, vitamin C, alpha tocopherol, pentoxiphillin in treatment of patients was tested against removing untoward effects of oxygen radicals formed during I/R and they were found effective. Those antioxidant substances activate antioxidant system and exert protective effect against end organ damage (Uysal et al. 2006). AST is a naturally forming carotenoid pigment and a potent biological antioxidant (Palozza et al. 1992). It shows oxygen radical scavengering activity and protects cell membranes, cells against lipid peroxidation and oxidative damage (Lim et al. 1992). In this study, lipid peroxidation was monitored with MDA measurement which arises from damage of cell membrane structures by free radicals. As expected MDA level in lung tissue was higher in I/R group compared to control and a decrease occurred in I/R+AST administered group compared to I/R group (Figure 1). In a study protective effect of AST in a kidney I/R model was investigated. They found an increase in MDA level in I/R group compared to sham and a decrease in I/R+AST group compared to I/R group (Qiu et al. 2015).
In another study focusing on protective effect of AST on I/R induced memory loss, MDA levels were increased in I/R group compared to sham group whereas a decrease in I/R+AST group compared to lone I/R administered group was determined (Xue et al. 2017). A similar increase in MDA level in lower I/R induced lung damage and a decrease due to melatonin administration was observed in another study (Uysal et al. 2006). In a study on rat aortic I/R investigating damage of this administration on lung damage, MDA level was increased in I/R group significantly (Kapan et al. 2009). Comparison of our data with those studies reveals comparable findings. When GSH level was evaluated, a decrease in I/R group compared to control (p<0.05) and an increase in this parameter in AST group compared to I/R group was determined. This increase was found statistically significant (p<0.05) (Figure 2). A study investigating effect of AST on liver I/R model also revealed a decrease in GSH in I/R group compared to control and an increase in I/R+AST group compared to I/R group (Curek et al. 2010). GSH level was ameliorated with AST in a study focusing on memory loss due to I/R (Xue et al. 2017). In another study concerning lung damage due to I/R and protective effect of silostazol and levosimendan, GSH levels were found significantly attenuated due to I/R (Önem et al. 2012). Our results were found similar with literature. I/R is an intensely studied subject due to its incidence however its pathogenesis and molecular pathways are not clarified completely. I/R damage occurs due to acute lung damage, acute kidney failure, shock and infection with different pathophysiological alterations in cellular, tissue and organ level. Investigation of extent of I/R damage can be conducted with histopathological evaluation of samples (Tassiopoulos et al. 1997; Wagner et al. 2002). Vascular congestion, epithelial loss and hyaline membrane formation, degenerations and necrosis in bronchiolar and alveolar cell walls, thickening in interalveolar septum, congestion in capillary walls, mononuclear cell infiltrations can be observed with light microscope (Odabasi, D.2006; Schnells et al. 1979; Pietra et al. 1981; Tanahashi et al. 1999). In addition extravascular erythrocytes, fibrinogen rich serum may fill alveolar and interstitial area thereby causing edema. Protein rich edema is caused by increased permeability in diffuse alveolar damage (Holter et al. 1986) and this permeability damage affects pulmonary surfactant system (Petty et al.1977; Aronsen et al. 1996). Alveolar surfactant is activated in the presence of plasma proteins. Anomaly or loss of this surfactant is ended up with micro atelectasis in alveoli and respiratory bronchioles (Holm et al. 1988). Such effects occurring during ischemia-reperfusion arise due to oxidative stress caused by augmented oxygen radical production in each stage of micro circulation (Colletti et al. 1995). Cells can adapt to a certain level of increased oxidative stress occurring in a slow increase fashion. However when oxidative level reaches to an intolerable level cellular function deteriorates and irreversible cell damage occurs which leads to cell death (Teoh et al. 2003). Lung tissue is one of the most vulnerable tissue against oxidative stress due to its special structure and function (Guo et al. 2007). Following I/R, formation of free oxidant radicals and inflammatory mediators initiate local and systemic damage (Grace, P.A. 1994). Although reperfusion of blood flow saves the extremity from necrosis, it may lead to multisystemic organ dysfunction and mortality. Local effects are observed in skeletal muscle and vessel endothelium whereas systemic effects are observed in especially myocardial tissue, lung and kidneys (Blaisdell et al. 2002). Lung tissue is the most vulnerable tissue for lower extremity I/R damage and due to occurred damage ventilator and inotropic support is needed in some cases which may even result in death (Rocker, G.M. 1997). Researchers administered some substances with antioxidant nature to minimize or prevent damage caused by I/R. In a study including 3 hours of I/R, widespread neutrophil infiltration, alveolar array disorientation, intraalveolar edema formation in lungs of I/R administered group. This serious lung damage was alleviated with dexamethaspinidin administration (Küçükebe, Ö.B. 2009). In a lower extremity I/R model dexametazon and aminoguаниdin administration decreased lung PNL numbers, interstitial edema and congestion compared to I/Rgroup (Tassiopoulos et al. 1997). Similarly in literature melatonin was given to alleviate damage following administration of different I/R models and PNL accumulation was attenuated due to melatonin administration (Celik et al. 2002; Inci et al. 2002). In histopathological investigation of protective effect of melatonin on lower extremity I/R induced lung damage; edema, alveolar congestion, presence of MNL cells, fibrin-platelet-trombus formation, chronic inflammation and intraalveolar bleeding in lungs were ameliorated by melatonin by preventing lipid peroxidation and reducing neutrophil infiltration (Uysal et al. 2006). Researchers formed ischemia in lungs by adjusting a clamp on abdominal aorta and following reperfusion congestion, mononuclear cell infiltration and interstitial edema which are frequently encountered in lung damage was avoided by using aprotin (Şirin et al. 2001). In a study investigating protective effect of caffeic acid phenylester in lower extremity I/R damage peribronchial and perivascular leucocyte infiltration was decreased compared to I/R administered group (Çalikoğlu et al. 2004). Similarly ascorbic acid was also found effective in preventing increase in MNL count, interstitial edema and congestion due to I/R (Berkan et al. 2001). Our study focuses on protective effect of AST in lung damage due to lower extremity I/R damage. Histopathological evaluation showed that I/R group had congestion in interalveolar capillaries, alveolar macrophage and erythrocyte accumulation in some
bronchiole and alveolar lumens, interstitial and alveolar edema, perivascular and peribronchiolar MNL cell infiltration, hyperemia in capillaries of inter alveolar septum, interstitial pneumonia with enlarged inter alveolar septum due to bleeding and inflammatory cellular infiltrations, degeneration and necrosis in some bronchioles and alveolar epithelial cells and desquamation with necrosis, muscular hyperplasia in vessel and bronchiolar smooth muscle layers, congestive atelectasis in different intensities due to heavy exudate accumulation in interstitial area and also disruption of normal morphological appearance of some alveolar in an emphysematous fashion was observed. However in I/R+AST group an alleviation of this damage was observed. Hyperemia in capillaries of some inter alveolar septum and inflammatory cellular infiltrations and an enlargement in some of the inter alveolar septum was observed but those findings was quite low compared to I/R group and resembling control group. Our results show similar findings with literature mentioned above when those histopathological findings and damage scores are evaluated together (Weiss et al. 1989; Faust et al. 1988; Feller et al. 1989).

**Conclusion:** The protective effect of AST has been demonstrated by biochemical, histopathological and immunohistochemical effects.

**Ethical Considerations:** Study was approved by Animal Experiments Local Ethical Committee (18.11.2015 - 2015/08, Decision number: 08/02).

**Conflict of Interest:** Authors declare no conflict of interest.

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Astaxanthin Ameliorates Lipopolysaccharide-Induced Neuroinflammation, Oxidative Stress and Memory Dysfunction through Inactivation of the Signal Transducer and Activator of Transcription 3 Pathway

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Abstract: Astaxanthin (AXT), a xanthophyll carotenoid compound, has potent antioxidant, anti-inflammatory and neuroprotective properties. Neuroinflammation and oxidative stress are significant in the pathogenesis and development of Alzheimer’s disease (AD). Here, we studied whether AXT could alleviate neuroinflammation, oxidative stress and memory loss in lipopolysaccharide (LPS) administered mice model. Additionally, we investigated the anti-oxidant activity and the anti-neuroinflammatory response of AXT in LPS-treated BV-2 microglial cells. The AXT administration ameliorated LPS-induced memory loss. This effect was associated with the reduction of LPS-induced expression of inflammatory proteins, as well as the production of reactive oxygen species (ROS), nitric oxide (NO), cytokines and chemokines both in vivo and in vitro. AXT also reduced LPS-induced β-secretase and Aβ1–42 generation through the down-regulation of amyloidogenic proteins both in vivo and in vitro. Furthermore, AXT suppressed the DNA binding activities of the signal transducer and activator of transcription 3 (STAT3). We found that AXT directly bound to the DNA binding domain (DBD) and linker domain (LD) domains of STAT3 using docking studies. The oxidative stress and inflammatory responses were not downregulated in BV-2 cells transfected with DBD-null STAT3 and LD-null STAT3. These results indicated AXT inhibits LPS-induced oxidant activity, neuroinflammatory response and amyloidogenesis via the blocking of STAT3 activity through direct binding.

Keywords: astaxanthin; lipopolysaccharide; Alzheimer’s disease; memory impairment; amyloidogenesis; neuroinflammation; oxidative stress; STAT3

1. Introduction

Alzheimer’s disease (AD) is an age-related neurodegenerative disorder associated with increased production and aggregation of amyloid beta (Aβ), an insoluble peptide, which causes oxidative damage and neuroinflammation in the brain [1,2]. The pathological abnormalities in AD include a profound loss of synapses, microglial activation and increased memory dysfunction [3,4].

Several studies have demonstrated that lipopolysaccharides (LPS)—the endotoxins produced by gram-negative bacteria—induce neuropathological and behavioural changes in mice that are similar...
to those produced in a human AD brain [5–7]. LPS generate a chronic inflammatory and oxidative stress in brain that result in the production and accumulation of Aβs in both the cerebral cortex and hippocampus [8–10]. The reactive oxygen species (ROS) released under stress cause lipid peroxidation, leading to the formation of products, such as malondialdehyde (MDA) and downregulates the levels of anti-oxidants, such as glutathione (GSH), aiding the development of AD [2,9]. Cytokines and chemokines, such as tumour necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β), interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-alpha (MIP-1α) and macrophage inflammatory protein 1-beta (MIP-1β) exert inflammatory response that promote the loss of synapses and cognitive dysfunction [6,11]. The pro-inflammatory cytokines and oxygen radicals have been found abundantly in the brains of patients with AD [12,13]. In addition, neuroinflammation and oxidative stress produced by LPS results in the activation of microglia within the hippocampus and entorhinal cortex [14].

Signal transducer and activator of transcription 3 (STAT3), the members STAT family of proteins play critical roles in inflammatory diseases, including AD [15,16]. The post-mortem analysis of hippocampus retrieved from AD patients as well as the analysis of the brains of AD mouse models have shown the activation and elevated levels of STAT3 [17]. In several studies, the level of phosphorylated STAT3 was demonstrated to have increased in LPS-induced neuroinflammation in vivo [18] and in vitro [19]. In the pathogenesis of AD, the activation of STAT3 signalling pathway is positively associated with cytokine signalling during neuronal differentiation and inflammation [17,20]. We previously found that in transgenic mice overexpressing IL-32, the phosphorylation of STAT3 was reduced, as were the levels of several cytokines, which reduced the memory impairment [21]. These data indicate that inhibition of STAT3 pathway and cytokine levels could be crucial for the reduction of memory impairment. The increased levels of ROS promote inflammatory processes and elevate the levels of pro-inflammatory cytokines [22]. Several studies have demonstrated that the phosphorylation of STAT3 is stimulated under oxidative stress [23–25]. In a previous study, we reported that STAT3 phosphorylation was upregulated by LPS-induced oxidative stress in astrocytes and microglial cells [24]. The depletion of GSH affected the phosphorylation of STAT3 in cardiac myocytes [25]. The oxidative stress-induced STAT3 phosphorylation could contribute to the development of several diseases. The blocking of STAT3 pathway protects against oxidative damages by increasing the levels of GSH [26]. Neuron-specific antioxidant oxidation resistance 1 delayed amyotrophic lateral sclerosis by delaying the activation of STAT3 [27]. These data indicate that oxidative stress could stimulate STAT3 and thus, accelerate the development of diseases. Moreover, STAT3 signalling was shown to trigger memory impairment and neuronal damage through the induction of neuroinflammation and oxidative stress [17,28].

Astaxanthin (AXT) is ubiquitous in nature, especially in the marine environment and is found in high amounts in the red-orange pigment of the shells of crustaceans (for example, crabs and, shrimp), salmon, trout and asteroidean [29]. It has been reported that AXT can protect skin from ultraviolet radiation-induced damages, ameliorate age-related macular degeneration, protect against chemically-induced cancers, increase high-density lipoproteins and enhance the immune system through its anti-oxidant and anti-inflammatory properties [29,30]. In previous studies, we have shown that anti-inflammatory and anti-oxidant agents prevent Aβ deposition by directly inhibiting the cleavage of amyloid precursor protein (APP) by γ-secretase [20,31–33], through the inhibition of STAT3 [34]. In the present study, we investigated whether AXT alleviates LPS-induced inflammatory response and memory impairment, both in vivo and in vitro.

2. Results

2.1. Astaxanthin Alleviates LPS-Induced Memory Impairment in Mice

Previous studies from our and other groups have demonstrated that memory impairment and amyloidogenesis can be induced by systemic injections of lipopolysaccharides (LPS) in in vivo
and in vitro models [20,32,33,35]. To investigate the memory-improving effects of AXT using the LPS-induced memory impairment model, we performed water maze and passive avoidance tests. The ability of mice to learn and recall spatial memory through escape latency and distance was investigated in water maze two times per day for 6 days. The average escape latency and escape distance at the end of training were about 18.33 s and 242.83 cm in the control group. The LPS-injected mice exhibited an average escape latency and escape distance to the platform of about 35.44 s and 546.81 cm in day 6. However, the LPS-injected mice that were given AXT on day 6 showed a dose-dependent and significant decrease in escape latency to 28.7 and 22.94 s and in escape distance to platform to 396.02 and 310.31 cm in the 30 and 50 mg/kg administration groups (Figure 1A,B). After the final day of the water maze test, we performed a probe test to calculate the time spent in the target quadrant for testing the maintenance of memory function. The average time spent in the target quadrant was decreased in the LPS-injected mice (22.83%) compared to that in the control group mice (29.00%) but administration of AXT in the memory impaired mice increased the average time spent in the target quadrant to 27.54% at AXT 30 mg/kg administration group (Figure 1C). Our results indicated that the LPS-injected mice required more time to find the hidden platform and performed fewer platform crossing compared to the mice in the control group. However, the AXT-administered mice required less time to find the hidden platform and performed increased platform crossing compared to the LPS-injected mice. We then evaluated as to how long the mice could remember, through the passive avoidance test. Although there were no significant differences in the training trial, the step-through latency in the LPS-injected mice (29.67 s) decreased in comparison to that in the control group (131.39 s). However, the step-through latency in the LPS-injected mice recovered to 51.40 s in the 30 mg/kg AXT administration group and to 108.26 s in the 50 mg/kg AXT administration group in the testing trial (Figure 1D). Overall, our results demonstrated that LPS-induced memory impairment was alleviated by AXT.

Figure 1. Effect of astaxanthin (AXT) on lipopolysaccharide (LPS)-induced improvement of memory impairment in the brain of mice. The mice (n = 8) were daily administrated AXT by oral gavage at dose of 30 or 50 mg/kg for 4 weeks. I.p. injection of LPS (250 μg/kg) was administrated except for control group on the 4th week for 7 days and they were evaluated for learning and memory of spatial information using the water maze. (A) Escape latency, the time required to find the platform and (B) escape distance, the distance swam to find the platform were measured. After the water maze test, (C) probe test to measure maintenance of memory were performed. The time spent in the target quadrant and target site crossing within 60 s was represented. (D) A passive avoidance test was performed by step-through method. n = 8 per group. The data are shown as the means ± SD of the mean. * p < 0.05 control group vs. LPS group, * p < 0.05 LPS-group vs. LPS with AXT group.
2.2. Astaxanthin Downregulates LPS-Induced Aβ Burden in the Brain of Mice

To investigate the association between memory improvement and in the reduction of Aβ deposition as a result of AXT administration, we measured the Aβ level in the brain. The Aβ level in the brains of LPS-injected mice (152%) were higher than the levels in the control group but it was decreased in the brains of AXT-administered mice (Figure 2A). We also measured the activity of β-secretase in the brain, because Aβs are produced by activated β-secretases. The activity of β-secretase was increased in the brains of LPS-injected mice (123%) compared to that in the brains of the control group mice but it was decreased in the brains of AXT-administered mice (Figure 2B). To confirm whether AXT could influence the inhibition of amyloidogenesis in the brain, we investigated the level of APP and β-secretase 1 (BACE1) proteins using western blot analysis. The expression levels of APP and BACE1 were observed to have increased in the brains of LPS-injected mice and the expression of APP was decreased in the 30 mg/kg AXT administration group and the expression of BACE1 was reduced by the administration of AXT (Figure 2C).

Figure 2. Effect of astaxanthin on LPS-induced Aβ accumulation and expression of amyloidogenic protein in the brain of mice. (A) The levels of Aβ1-42 in the brain of mice were assessed using a specific Aβ ELISA. n = 4 per group (B) The β-secretase activity in the brain of mice was measured using assay kit. n = 4 per group (C) The expression of APP and BACE1 were detected by western blot using specific antibodies in the brain of mice. β-actin protein was used as an internal control and graphs represented the arbitrary density of blot signal. n = 4 per group. The data are shown as the means ± SD of the mean. # p < 0.05 control group vs. LPS group, * p < 0.05 LPS-group vs. LPS with AXT group.

2.3. Astaxanthin Prevents LPS-Induced Neuroinflammation in the Brain of Mice

The activation of microglia is implicated in the neuroinflammation during the development of AD. To investigate the protective effect of AXT on the activation of astrocytes and microglia, we performed immunohistochemistry to detect the expression of glial fibrillary acidic protein (GFAP) (a marker protein of astrocytes), IBA-1 (a marker protein of microglia cells) and inflammatory proteins (iNOS and COX-2) in the CA3 and DG (dentate gyrus) regions of the brain of mice. The number of GFAP and IBA-1-reactive cells were lower in the AXT-administered mice compared to that in the LPS-injected mice, which was much higher than the number in the control group mice (Figure 3A). The number
of iNOS and COX-2-reactive cells was also reduced in the AXT-administered mice compared to that in the LPS-injected (Figure 3B). The expression levels of GFAP, IBA-1, iNOS and COX-2 were further evaluated using western blot analysis. In consonance with the immunohistochemistry results, the increased expressions levels of these proteins by LPS were decreased in the AXT-administered mice (Figure 3C). However, the expression of GFAP was decreased at 30 mg/kg in the AXT-administered mice (Figure 3C). The production of pro-inflammatory cytokines is also involved in neuroinflammation and enhances the development of AD [36]. The pro-inflammatory cytokines also enhance the APP production and the process of proteolytic cleavage of APP to increase the production of Aβ [37]. To examine the production of a variety of pro-inflammatory cytokines and chemokines in the brain of mice, we performed real-time PCR. The levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and of chemokines, such as MCP-1, MIP-1α and MIP-1β were increased in the LPS-injected mice but their levels were decreased in the AXT-administered mice (Figure 4A,B).

**Figure 3.** Effect of astaxanthin on LPS-induced neuroinflammation and amyloidogenesis in the brain of mice. Immunohistochemical analysis of (A) glial fibrillary acidic protein (GFAP), IBA-1 (B) iNOS and COX-2 antibodies were investigated two different regions (CA3, cornu ammonis 3 and DG; dentate gyrus) in 20-μm-thick sections of the brain hippocampus of mice with specific primary antibodies and the biotinylated secondary antibodies. (scale bars, 100 μm) n = 3 per group (C) The expression of GFAP, IBA-1, iNOS and COX-2 were detected by western blot using specific antibodies in the brain of mice. β-actin protein was used as an internal control and graphs represented the arbitrary density of blot signal. n = 4 per group. The data are shown as the means ± SD of the mean. * p < 0.05 control group vs. LPS group, * p < 0.05 LPS-group vs. LPS with AXT group.
Figure 4. Effect of astaxanthin on LPS-induced pro-inflammatory cytokines, chemokines and oxidative stress in the brain of mice. mRNA expression levels of (A) pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and chemokines (B) MCP-1, MIP-1α and MIP-1β in the brain of mice were measured using quantitative real-time RT-PCR (reverse transcription polymerase chain reaction). (C) GSH/GSSG ratio, total GSH and TBARS levels in the brain of mice were assessed using assay kit. n = 4 per group. The data are shown as the means ± SD of the mean. # p < 0.05 control group vs. LPS group, * p < 0.05 LPS-group vs. LPS with AXT group.

2.4. Astaxanthin Reduces LPS-Induced Oxidative Stress in the Brain of Mice

Brain is particularly vulnerable to oxidative stress because of its high utilization of oxygen; therefore, oxidative stress has a crucial role in the pathogenesis of AD. Aβ may induce the production of ROS and ROS-related neurotoxic factors and thereby destroy the various types of biological molecules [9]. We determined the levels of oxidative stress in the brain of mice. Reduced glutathione (GSH) is a vital endogenous protective antioxidant against oxidative stress, which is oxidized to GSSG, referred to as the oxidized glutathione; the GSH/GSSG ratio and total GSH level are indices of the protective ability of cells under oxidative stress induced by toxicants. The GSH/GSSG ratio and total GSH level was observed to decrease in the LPS-injected mice compared to their values in the control group mice but it was increased by AXT treatment (Figure 4C). However, the level of thiobarbituric acid—a marker of lipid peroxidation—was elevated by LPS but was reduced by AXT treatment (Figure 4C).

2.5. Astaxanthin Inhibits Amyloidogenesis and Neuroinflammation in Microglia BV-2 Cells

Microglial cells are the primary LPS-responsive cells in the central nervous system (CNS). We performed western blot analysis, real-time PCR, β-secretase activity assay and nitro oxide and oxidative stress assays in BV-2 microglial cells to elucidate the neuroprotective, anti-inflammatory and anti-oxidant roles of AXT against LPS-induced neuroinflammation. The expression levels of APP and BACE1 protein were higher in response to LPS but these expression levels were inhibited by AXT
treatment (Figure 5A). The LPS-induced β-secretase activity was decreased in the AXT-treated BV-2 cells at 20 μM (Figure 5B). We also detected the expression levels of iNOS, COX-2 and IBA-1 by western blot analysis. The expression levels of iNOS, COX-2 and IBA-1 were reduced in the AXT-treated BV-2 cells in a concentration-dependent manner (Figure 5C). We also determined the production of pro-inflammatory cytokines and chemokines in the BV-2 cells by real-time PCR. The levels of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and chemokines such as MCP-1, MIP-1α and MIP-1β were increased by LPS but were decreased in the AXT-treated BV-2 cells (Figure 6A,B).

Figure 5. Effect of astaxanthin on amyloidogenesis and neuroinflammation in the microglia cell. Microglia BV-2 cells were treated with LPS (1 μg/mL) and AXT (5, 10 and 20 μM). (A) The expression of APP and BACE1 were detected by western blot using specific antibodies in the microglia BV-2 cells. β-actin protein was used as an internal control and graphs represented the arbitrary density of blot signal. (B) The levels of β-secretase activity in the microglia BV-2 cells were assessed using assay kit. (C) The expression of iNOS, COX-2 and IBA-1 were detected by western blot using specific antibodies in the microglia BV-2 cells. β-actin protein was used as an internal control and graphs represented the arbitrary density of blot signal. n = 3 per group; means ± SD of the mean. # p < 0.05 control group vs. LPS group, * p < 0.05 LPS-group vs. LPS with AXT group.
2.6. Astaxanthin Inhibits LPS-Induced Oxidative Stress in the Microglia BV-2 Cells

Neuroinflammatory diseases, such as AD, are characterized by the reaction of superoxides with nitric oxide [38]. To determine whether AXT could reduce oxidative stress in microglia and could be used as a novel inhibitor, we determined the NO, total GSH and TBARS levels in the BV-2 cells. The concentration of NO was increased by LPS but it was decreased in the AXT-treated BV-2 cells (Figure 6C). The total GSH level was increased by LPS and was decreased by AXT treatment. However, there is no big difference between each group (Figure 6C). In addition, the level of TBARS was elevated by LPS but was decreased by AXT (Figure 6C).

2.7. Astaxanthin Inhibits the Phosphorylation of STAT3 in the Brain of Mice and the Microglia BV-2 Cells

To determine the involvement of the STAT3 pathway in the inhibitory effect of AXT, we investigated the interaction between AXT and STAT3. In the presence of AXT, the LPS-induced phosphorylation of STAT3 was inhibited in the LPS-injected mice at 50 mg/kg (Figure 7A). In addition, the LPS-induced STAT3 activation was reduced by AXT in the BV-2 cells (Figure 7B). The inhibition of phosphorylated STAT3 by STAT3 inhibitor was more effective than by AXT (Figure 7C). Moreover, the levels of the phosphorylated STAT3 showed a larger decrease when the BV-2 cells were co-treated with AXT and STAT3 inhibitor (Figure 7C).
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Figure 7. A vital role of STAT3 by binding astaxanthin LPS-induced neuroinflammation and oxidative stress in in vivo and in vitro. The expression of p-STAT3 and STAT3 were detected by western blot using specific antibodies in (A) the brain of mice (B) the microglia BV-2 cells. β-actin protein was used as an internal control and graphs represented the arbitrary density of blot signal. (C) The expression of p-STAT3 and STAT3 were detected by western blot using specific antibodies in the microglia BV-2 cells.

2.8. Astaxanthin Directly Binds to the DBD and LD of STAT3

Astaxanthin showed inhibitory effects on STAT3 activation, both in vivo and in vitro. We investigated whether this could be associated with the physical interaction of AXT and STAT3. We found the potential binding mode of AXT to STAT3 and the precise conformation of AXT at the binding site in STAT3 via virtual docking analysis. Virtual docking analysis was performed using AutoDock VINA [39] and molecular graphics for the best binding model was generated using Discovery Studio Visualizer 2.0 (BIOVIA, San Diego, CA, USA). The docking model showed a surface rendering of the DNA-binding domain (DBD) (inside a binding pocket comprised of Ile258, Gln247, Ala250, Cys251, Glu324, Arg325 and Gln326) and linker domain (LD) (inside a binding pocket comprised of Asp334, Thr515, Lys573, Leu577, Ala578 and Lys574) of STAT3 with AXT; the binding affinity between the two was found to be -9.0 kcal/mol. To elucidate the interaction between AXT and the DBD and LD of STAT3, we performed a pull-down assay using AXT-conjugated epoxy-activated Sepharose 6B beads and cell lysate transfected with DBD-null STAT3 and LD-null STAT3 (Figure 8A). The AXT-conjugated beads pulled down the STAT3, whereas the vehicle control beads could not do so in the pull-down assay (Figure 8B). The luciferase activity of STAT3 was increased in the BV-2 cells transfected with wild-type STAT3, DBD-null STAT3 and LD-null STAT3 vectors by LPS. However, the AXT treatment decreased the STAT3 luciferase activity in the BV-2 cells transfected with wild-type STAT3 vector; however, the luciferase activity of STAT3 was not affected by the AXT treatment in the BV-2 cells transfected with the DBD-null STAT3 and LD-null STAT3 vectors (Figure 8C). In addition, the expression levels of APP and p-STAT3 proteins were downregulated in the BV-2 cells transfected with wild-type STAT3 vector; however, the expression levels of these protein were more decreased in
the BV-2 cells transfected with wild-type STAT3 vector than in the BV-2 cells transfected with DBD-null STAT3 and LD-null STAT3 vectors and treated with AXT (Figure 8D). We also examined the production of pro-inflammatory cytokines (TNF-α and IL-6) and the ROS level (NO and TBARS) in the BV-2 cells transfected with wild-type STAT3, DBD-null STAT3 and LD-null STAT3 vectors. The levels of TNF-α and IL-6 were decreased by AXT in the BV-2 cells transfected with the wild-type STAT3 but these cytokine levels were not affected by the AXT treatment in the BV-2 cells transfected with the DBD-null STAT3 and LD-null STAT3 vectors (Figure 8E). The concentration of NO and TBARS were also reduced in the BV-2 cells transfected with the wild-type STAT3. However, the ROS levels were not inhibited by AXT in the BV-2 cells transfected with DBD-null STAT3 and LD-null STAT3 vectors (Figure 8F). Thus, AXT could contribute to the suppression of the STAT3 activity by binding to the DBD and LD domains of STAT3.

3. Discussion

Increasing evidence demonstrates that AXT possesses anti-oxidant and anti-inflammatory activities that could ameliorate the pathogenesis of AD [40,41]. In the present study, we showed
that AXT could alleviate the LPS-induced neuroinflammation and oxidative stress resulting in the decrease in Aβ levels and in the activity of β-secretase in AXT-administered mice as well as in AXT-treated BV-2 microglia cells. The anti-oxidative, anti-inflammatory and anti-amyloidogenic effects were associated with the inhibitory effect of AXT on the memory impairment.

The pathogenesis of AD is critically associated with neuroinflammation in the brain [36]. Neuroinflammation occurs in the vulnerable regions of AD brain where there is high deposition of Aβ [42]. iNOS and COX-2 are known to be involved in inflammatory response [13,36]. iNOS is upregulated in the brain of patients with AD and iNOS knockout (KO) is protective in mouse models of AD [36,43]. COX-2 is also increased in the brain of AD patients [44,45]. In the brain of LPS-injected mice and LPS-treated BV-2 microglial cells, the immunoreactivity and expression of iNOS and COX-2 were increased compared to that in the control group; however, the expression of these proteins was decreased in the AXT-administered mice and AXT-treated BV-2 microglial cells. The astrocytes and microglial cells are activated and accelerate the progression of neurodegenerative diseases. In the brain of LPS-injected mice and LPS-treated BV-2 microglial cells, the immunoreactivity and expression of GFAP and IBA-1 were decreased in the AXT-administered mice and AXT-treated BV-2 microglial cells. The activation of astrocytes and microglial cells also releases a series of damaging cytokines and chemokines [46]. Cytokines contribute to nearly every aspect of neuroinflammation, including pro-inflammatory processes, chemotaxis and response of microglial cells to Aβ deposits [36]. Chemokines have been found to be upregulated in the brain of AD patients and recruit the astrocytes and microglial cells to the sites of Aβ deposition [47]. In the brain of LPS-injected mice and LPS-treated BV-2 microglial cells, TNF-α, IL-1β and IL-6 were also upregulated compared to their levels in the control group. However, these cytokines were downregulated, both in vivo and in vitro. MCP-1, MIP-1α and MIP-1β were also increased in the brain of LPS-injected mice and LPS-treated BV-2 cells compared to their levels in the control group; however, their levels were decreased in the AXT-administered mice and AXT-treated BV-2 microglial cells. These data indicate that the anti-inflammatory effects of AXT could contribute to the reduction in amyloidogenesis and thereby, memory impairment.

The CNS is particularly vulnerable to oxidative stress because of a high oxygen consumption rate, abundant unsaturated lipids and a relative deficit of antioxidant enzymes compared to other organs [9,48]. The oxidative stress may damage the various types of biological molecules, such as GSH and antioxidant enzymes [9]. Lipid peroxidation appears to be more marked in the AD patients. The end products of peroxidation, such as malondialdehyde and peroxy nitrite, are also increased in the brain of patients with AD [49]. Moreover, oxidative stress can transform the non-aggregated Aβ into aggregated Aβ [50]. Aβ also stimulates ROS production in the microglia in rodents [51]. For example, lipid and protein peroxidation is increased in the APP/PS1 double knock-in mice [52]. The level of GSH was decreased in the APPsw/PS1dE9 double transgenic mice but the level of MDA was increased in the APPsw/PS1dE9 double transgenic mice [9]. In the present study, the GSH/GSSG ratio and the total GSH level were observed to recover and the TBARS level was decreased by AXT administration. Moreover, the NO level was also decreased in the AXT-treated microglial cells compared to its level in the LPS-treated BV-2 microglial cells. These data also indicate that the anti-oxidant property of AXT could be significant for reduction of amyloidogenesis and could, thus, reduce memory dysfunction induced by LPS.

STAT3 is abundantly expressed in brain and participates principally in the regulation of genes involved in inflammation [53]. In the neuronal compartment, the activation of STAT3 has been observed in senile plaques and it is involved in the neuronal loss by apoptosis in the brain [54]. LPS could upregulate neuroinflammation in microglia cells through the activation of STAT3 [55]. Pro-inflammatory cytokines affect the phosphorylation of STAT3 in transgenic AD mouse model [21,56]. Especially, IL-6 is known to activate STAT3, which is associated with memory impairment in the LPS-injected IL-6 KO mice [57]. Our results indicated that the expression of p-STAT3 was increased in LPS-injected mice and LPS-treated BV-2 microglia cells but these increases were prevented by AXT.
These data indicate that the inhibitory effect of AXT on the STAT3 pathway could be significant for the anti-inflammatory and anti-oxidant effects of AXT and could, thus, be associated with the memory impairing effects of AXT.

Our results indicate that AXT plays an inhibitory role in LPS-induced neuroinflammation through the inactivation of STAT3. We investigated whether AXT could inhibit the STAT3 activity through binding of AXT and STAT3. Molecular docking simulations revealed that AXT directly binds to the DBD and LD domains of STAT3. As shown by luciferase and pull-down assays, the DBD-null STAT3 and LD-null STAT3 abolished the inhibitory effect of AXT on STAT3 activation. These data suggest that the DBD and LD are crucial for the AXT STAT3 interaction and imply that AXT may exert anti-inflammatory and anti-oxidative effects via the inhibition of STAT3 by directly binding to the DBD and LD domains. In conclusion, we demonstrate that AXT protects against LPS-induced mice AD model by inhibiting of the STAT3 activity, which could result in the inhibition of Aβ accumulation by attenuation of β-secretase activity through anti-oxidative and anti-neuroinflammatory properties. We, therefore, suggest that the formulation and synthetic modification of AXT could provide a potent therapeutic agent for the prevention of AD.

4. Materials and Methods

4.1. Animals Experiment and Housing Condition

Eight weeks old male imprinting control region (ICR) mice were purchased from DBL (Eumsung, Korea). The experiment was performed in accordance with the guidelines proscribed by the Chungbuk National University Animal Care Committee (CBNUA-929-16-01). The mice were acclimatized to the laboratory environment, maintained at 22 ± 1 °C and relative humidity of 55 ± 10%, with 12 h light-dark cycles throughout the experiment. All mice were fed a standard laboratory chow diet ad libitum. All mice were randomly divided into the following four groups (n = 8/group): control group, LPS group, LPS with AXT 30 mg/kg group, LPS with AXT 50 mg/kg group. The mice from AXT groups were daily administrated AXT that dissolved in olive oil for 4 weeks by oral gavage. Intraperitoneal (i.p.) injection of LPS (250 μg/kg) was administered to all groups except for the control group on the 4th week for 7 days. Control mice were given an equal volume of vehicle instead. Subsequently, the behavioural tests of learning and memory capacity were assessed using the water maze, probe and passive avoidance test after AXT/LPS administration. Mice were sacrificed after behavioural tests by CO2 asphyxiation.

4.2. Morris Water Maze

The water maze test is a widely accepted method for examining cognitive function and we performed this test as described by Morris et al. [58]. Maze testing was fulfilled by the SMART-CS (Panlab, Barcelona, Spain) program and equipment. A circular plastic pool (height: 35 cm, diameter: 100 cm) was filled with squid ink water kept at 22–25 °C. An escape platform (height: 14.5 cm, diameter: 4.5 cm) was submerged 1–1.5 cm below the surface of the water in position. The test was performed two times a day for 6 days during the acquisition phase, with two starting points of rotational starts. The position of the escape platform was kept constant. Each trial lasted for 60 s or ended as soon as the mouse reached the submerged platform. Escape latency and escape distance of each mouse were monitored by a camera above the centre of the pool connected to a SMART-LD program (Panlab, Barcelona, Spain). A quiet environment, consistent lighting, constant water temperature and a fixed spatial frame were maintained throughout the experimental period.

4.3. Probe Test

To assess memory consolidation, a probe test was performed 24 h after the water maze test. For the probe test, the platform was removed from the pool which was used in the water maze test and the mouse were allowed to swim freely. The swimming pattern of each mouse was monitored
and recorded for 60 s using the SMART-LD program (Panlab, Barcelona, Spain). Consolidated spatial memory was estimated by the time spent in the target quadrant area.

4.4. Passive Avoidance Test

The passive avoidance response was determined using a “step-through” apparatus (Med Associates Inc., Fairfax, VT, USA) that is divided into an illuminated compartment and a dark compartment (each 20.3 × 15.9 × 21.3 cm) adjoining each other through a small gate with a grid floor, 3.175 mm stainless steel rods set 8 mm apart. 48 h after the probe test, a training trial was performed. The mice were placed in the illuminated compartment facing away from the dark compartment for the training trial. When the mice moved completely into the dark compartment, it received an electric shock (0.45 mA, 3 s duration). Then, the mice were returned to their cage. 24 h after the training trial, each mouse was placed in the illuminated compartment and the latency period to enter the dark compartment defined as “retention” was measured. The time when the mice entered into the dark compartment was recorded and described as step-through latency. The retention trials were set at a cut-off time limit of 180 s.

4.5. Collection and Preservation of Brain Tissue

After the behavioural tests, mice were anaesthetized with CO₂ gas and then perfused with phosphate-buffered saline (PBS). The brains were immediately removed from the skull and the tissues were divided in half. One stored at −80 °C, the other was fixed in 4% paraformaldehyde for 72 h at 4 °C. The brains were transferred to 30% sucrose solutions, respectively.

4.6. Microglial BV-2 Cells Cultures

Microglial BV-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Microglial BV-2 cells were maintained with serum-supplemented culture media of DMEM supplemented with FBS (10%) and antibiotics (100 units/mL). The microglial BV-2 were incubated in the culture medium in a humidified incubator at 37 °C and 5% CO₂. The cultured cells were treated with several concentrations (5, 10, 20 μM) of AXT, 3 h before LPS (1 μg/mL) addition. The cells were harvested after 24 h.

4.7. Western Blot Analysis

Homogenized brain tissues were lysed by protein extraction solution (PRO-PREP, iNtRON, Sungnam, Korea) and the total protein concentration was determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). 40 μg of extracted protein were separated by SDS/PAGE and transferred to Immobilon® PVDF membranes (Millipore, Bedford, MA, USA). The membrane was blocked with 5% dried skimmed milk for 1 h at room temperature, followed by incubation with specific primary antibodies for overnight at 4 °C. The membranes were washed with Tris-buffered saline containing 0.05% Tween-20 (TBST) and incubated with diluted horse radish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washes, the binding of antibodies to the PVDF membrane was detected using the Immobilon Western Chemilum HRP substrate (Millipore, Bedford, MA, USA). The band intensities were measured using the Fusion FX 7 image acquisition system (Vilber Lourmat, Eberhardzell, Germany) and quantified using Image J software. Specific primary antibodies were purchased from Santa Cruz Biotechnology (GFAP, p-STAT3, STAT3 and β-actin; Dallas, TX, USA), Cell signalling Technology (iNOS and COX-2; Trask Lane, Danvers, MA, USA) and Abcam (APP, BACE1 and IBA-1; Cambridge, MA, USA). Secondary antibodies were purchased from Santa Cruz Biotechnology (anti-mouse, anti-rabbit and anti-goat; Dallas, TX, USA).
4.8. Immunohistochemistry

After being transferred to 30% sucrose solutions, brains were cut into 20 μm sections by using a cryostat microtome (Leica Microsystems, Seoul, Korea). After two washes in PBS (pH 7.4) for 10 min each, endogenous peroxidase activity was quenched by incubating the samples in 3% hydrogen peroxide in PBS for 20 min. The sections were blocked for 1 h in 5% bovine serum albumin (BSA) solution and incubated with specific primary antibodies for overnight at 4 °C. And then, the sections were washed twice for 10 min each in PBS and incubated in biotinylated anti-mouse or anti-rabbit or anti-goat IgG-horseradish peroxidase (HRP) secondary antibodies for 90 min. The sections were washed three times for 10 min each in PBS and visualized by a chromogen DAB (Vector Laboratories) reaction for up to 10 min. Finally, the sections were dehydrated in ethanol, cleared in xylene, mounted with Permount (Fisher Scientific, Hampton, NH) and evaluated on a light microscope (Nikon, Tokyo, Japan) and the quantitated positive cells manually. We investigated the region of brain from CA3 and DG region in hippocampus for anatomical studies. CA3 and DG network is the most critical for contributing to memory storage and retrieval of memory sequences. Specific primary antibodies were purchased from Santa Cruz Biotechnology (GFAP; Dallas, TX, USA) Cell signalling Technology (iNOS and COX-2; Trask Lane, Danvers, MA, USA) and Abcam (IBA-1; Cambridge, MA, USA).

4.9. Measurement of Aβ1–42

Lysates of brain tissue were obtained through protein extraction buffer containing protease inhibitor and were centrifuged at 14,000 rpm for 30 min to extract protein. Aβ1–42 levels were determined using each specific mouse amyloid-beta peptide 1-42 ELISA Kit (CUSABIO, Carlsbad, CA, USA). In brief, 100 μL of sample was added into a precoated plate and incubated for 2 h at 37 °C. After removing any unbound substances, a biotin-conjugated antibody specific for Aβ1–42 was added to the wells. After washing, avidin-conjugated HRP was added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution was added to the wells and the colour was developed in proportion to the amount of Aβ1–42 bound in the initial step. The the colour was development was stopped and the intensity of the colour was measured.

4.10. Assay of β-Secretase Activities

β-secretase activity in the mouse brains was determined using a commercially available β-secretase activity kit (Abcam, Cambridge, MA, USA). Solubilized membranes were extracted from brain tissues using β-secretase extraction buffer, incubated on ice for 1 h and centrifuged at 5000 × g for 10 min at 4 °C. The supernatant was collected. A total of 50 μL of sample (total protein 100 μg) or blank (β-secretase extraction buffer 50 μL) was added to each well (used 96-well plate) followed by 50 μL of 2 × reaction buffer and 2 μL of β-secretase substrate incubated in the dark at 37 °C for 1 h. Fluorescence was read at excitation and emission wavelengths of 335 and 495 nm, respectively, using a fluorescence spectrometer (Gemini EM, Molecular Devices, CA, USA).

4.11. RNA Isolation and Quantitative Real-Time RT-PCR

Total RNA from brain tissues were extracted by RiboEx™ Total RNA isolation solution (GeneAll Biotechnology, Seoul, Korea) and cDNA was synthesized using a High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) for custom-designed primers and β-actin was used for house-keeping control using a QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany). Cycling conditions consisted of a denaturation of 5 s at 95 °C and a combined annealing/extension of 10 s at 60 °C followed by 40 cycles. The values obtained for the target gene expression were normalized to β-actin and quantified relative to the expression in control samples.
4.12. Nitro Oxide and Oxidative Stress Assay

Nitro oxides (NO) were measured according to the manufacturer’s protocol (iNtRON, Sungnam, Korea). Hydrogen peroxides assay was performed as described in the manufacturer’s protocol (Cell Biolabs, San Diego, CA, USA). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were measured using GSH/GSSG Ratio Detection Assay Kit (Abcam, Cambridge, MA, USA). Lipid peroxidation was measured by determining the generation of malondialdehyde (MDA; TBARS Assay kit, Cayman, Ann Arbor, MI, USA).

4.13. Plasmid Construction

The coding region of Mus musculus STAT3 was amplified by PCR using full-length M. musculus STAT3 cDNA as a template. Purified PCR products were double-digested with EcoRI and XhoI and then sub-cloned into the pcDNA3.1 vector containing a cytomegalovirus promoter, pUC origin and an ampicillin-resistance gene. STAT3 deletion mutant (DBD deletion, LD deletion) was generated by Bionics (Seoul, Korea) and the mutants were checked by sequencing.

4.14. Pull-Down Assay

AXT (1 mg) was dissolved in 1 mL of coupling buffer (0.1 M NaHCO3, pH 11.0 containing 0.5 M NaCl) and conjugated with epoxy-activated Sepharose 6B (GE Healthcare Korea, Seoul, Korea). The epoxy-activated Sepharose 6B was swelled and washed in distilled water on a sintered-glass filter and then washed with the coupling buffer. The epoxy-activated Sepharose 6B beads were added to the AXT-containing coupling buffer and rotated at 4 °C overnight. After washing, unoccupied binding sites were blocked with 0.1 M Tris-HCl (pH 8.0) for 2 h at room temperature. The AXT-conjugated Sepharose 6B was washed with three cycles of alternating pH wash buffers (buffer 1: 0.1 M acetate and 0.5 M NaCl, pH 4.0; buffer 2: 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0). The control unconjugated epoxy-activated Sepharose 6B beads were prepared as described above in the absence of AXT. The cell lysate was mixed with AXT-conjugated Sepharose 6B or with Sepharose 4B at 4 °C overnight. The beads were then washed three times with TBST. The bound proteins were eluted with SDS loading buffer. The proteins were resolved by SDS-PAGE followed by immunoblotting with an antibody against STAT3 (1:1000 dilutions, Santa Cruz Biotechnology).

4.15. Statistical Analysis

The data were analysed using the GraphPad Prism 4 version 4.03 software (Graph-Pad Software, La Jolla, CA, USA). Data are presented as mean ± SD. When the P value in the analysis of variance test indicated statistical significance, the differences were assessed by the Tukey’s test. A value of p ≤ 0.05 was considered to be statistically significant.

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Full paper

Anatomy

Running head: ASTAXANTHIN IN RAT AFLATOXICOSIS

Title: The effects of astaxanthin on liver histopathology and expression of superoxide dismutase in rat aflatoxicosis

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ABSTRACT

The metabolism of aflatoxin B$_1$ (AFB$_1$) generates reactive oxygen species (ROS) that destroys hepatocytes. Meanwhile, astaxanthin (AX) is known to have stronger antioxidative activity than other carotenoids. This study aimed to investigate hepatoprotective role of AX from AFB$_1$-induced toxicity in rat by histopathological study and immunohistochemistry of Cu/Zn-SOD (SOD1) which acts as the first enzyme in antioxidative reaction against cell injury from ROS. Twenty Wistar rats were randomly divided into 4 groups. The control and AFB$_1$ groups were gavaged by water for 7 days followed by a single DMSO and 1 mg/kg AFB$_1$, respectively. The AXL+AFB$_1$ and AXH+AFB$_1$ groups were given of 5 mg/kg and 100 mg/kg AX for 7 days before 1 mg/kg AFB$_1$ administration. The result showed significantly elevated liver weight per 100 g body weight in AFB$_1$ group. The histopathological finding revealed vacuolar degeneration, necrosis, megalocytosis and binucleation of hepatocytes with bile duct hyperplasia in AFB$_1$ group. The severities of pathological changes were sequentially reduced in AXL+AFB$_1$ and AXH+AFB$_1$ groups. Most rats in AXH+AFB$_1$ group owned hypertrophic hepatocytes and atypical proliferation of cholangiocytes which are adaptive responses to severe hepatocyte damage. The SOD1 expression was also significantly higher in AXH+AFB$_1$ group than solely treated AFB$_1$ and AXL+AFB$_1$ groups. In conclusion, AX alleviated AFB$_1$-induced liver damage in rat by stimulating SOD1 expression and transdifferentiation of cholangiocytes in dose dependent manner.

Key words: aflatoxin B$_1$, astaxanthin, histopathology, immunohistochemistry, superoxide dismutase
Aflatoxin is a group of mycotoxins which are produced by Aspergillus spp., especially A. flavus, A. parasiticus and A. nomius [18]. This health hazardous toxin can be found as a contaminant in agricultural commodities during cultivation, harvesting, transport and storage in various regions with hot and humid climates [2, 18, 47]. Aflatoxin B$_1$ (AFB$_1$) is the most potent hepatotoxic agent which causes histopathological changes of liver such as necrosis, vacuolar degeneration, fatty changes, megalocytosis and binucleation of hepatocyte. Furthermore, bile duct hyperplasia and periportal fibrosis are observed [1, 2, 48, 49]. During AFB$_1$ metabolism activated by cytochrome P450, the reactive oxygen species (ROS), such as superoxide ion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are produced excessively leading to oxidative stress that could be eradicated by antioxidants [27, 44]. However, the overwhelming oxidative stress beyond an ability of antioxidants to handle results in damage of critical macromolecules (lipid, DNA and protein) and loss of biological functions such as calcium influx, membrane leakage and consequently affected DNA stability that leads to cancer [21, 25, 41]. The oxidative damage can be protected by enzymatic antioxidants, including superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), and non-enzymatic mechanisms by low molecular mass compounds such as glutathione, ascorbic acid, α-tocopherol and carotenoids [15]. The function of enzymatic antioxidants is to prevent the ROS from attacking other essential proteins in the cell by converting oxidative products into non-dangerous molecules by multi-step processes, while the activity of non-enzymatic antioxidants is to interrupt free radical chain reactions and neutralize the ROS in a process called radical scavenging [31]. One of the key anti-oxidative enzymes, SODs, is a family of enzymes that are important for the biological defense of cell injuries mediated through oxygen free radicals. The three main SOD isoforms have been identified in mammalian cells with different localization: the cytosolic Cu/Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2) and extracellular Cu/Zn -SOD (SOD3). They
detoxify superoxide ion and change into hydrogen peroxide which is inactivated by CAT into oxygen and water which are not harmful for cells. In hepatocyte, SOD1 expression is in the cytoplasm, followed by inside the nucleus. Its distribution was clarified to overlap with the site of superoxide production. High concentration of SOD1 was found in lysosome by nonselective autophagy since it is resistant to protease digestion. Surprisingly, the endoplasmic reticulum (ER) does not contain remarkable concentrations of SOD1 because the production of superoxide is on the cytoplasmic side which towards the surrounding cytoplasm whereas it is very low inside the ER cisternae. In mitochondria, the superoxide is formed by electron released from electron transport chain, but SOD2 plays major protective role in this part instead of SOD1[6, 9].

Astaxanthin (AX) (3,3′-dihydroxy-β, β-carotene-4,4′-dione) is a red carotenoid pigment found in microorganisms such as the green algae Haematococcus pluvialis [36], the red yeast Phaffia rhodozyma [7], and in many marine animals [43]. It has been used as a feed supplement to promote growth, egg quality, muscle and yolk pigmentation, immune system and antioxidant capacity in livestock, especially aquatic animals and poultry [35, 43]. AX has been reported as a powerful non-enzymatic antioxidant for scavenging variety of free radicals. Its antioxidative activity is 10 times stronger than zeaxanthin, lutein, tunaxanthin, canthaxanthin and β-carotene, and 100 times greater than α-tocopherol [26, 29]. AX can protect acute CCl₄ induced liver damage by inhibiting lipid peroxidation and increasing antioxidative mechanism by stimulating glutathione and SOD activities [20]. It prevents liver fibrosis by suppressing multiple profibrogenic factors in mice [39]. Moreover, AX also has anti-inflammatory activity by suppressing the level of NF-kB which regulates the production of inflammatory mediators [22]. Although AX has been widely studied for its hepatoprotective property, the investigation of AX activity on liver protection prior to an acute AFB₁ administration in rat has never been studied. In present study, we focused on histopathological changes and immunohistochemistry of SOD1 in
AFB₁ intoxicated rats by comparing the two doses of 5 mg/kg or 100 mg/kg AX. Since the climate in Southeast Asia is suitable for the growth of *Aspergillus* spp., contamination of AFB₁ has been found frequently during the processes of human and animal food production. Therefore, this study will be beneficial for the preventive treatment of aflatoxicosis in human and animal in the future.

MATERIALS AND METHODS

**Animals**

A total of 20 male Wistar rats, 6-week-old, weighing 200-250 g were obtained from National Laboratory Animal Center (Nakhon Pathom, Thailand). All rats were acclimated at 22-25°C in 12 hr dark/light cycle and fed *ad libitum*. This study has been approved by the Institutional Animal Care and Use Committee of Kasetsart University, Thailand (ID# ACKU 61-VET-023).

**Chemicals and preparation**

Aflatoxin B₁ (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 50% (w/v) DMSO in distilled water to the final concentration of 1 mg/kg and fed for each rat [34]. AstaMax®10S (Guangzhou Juyuan Bio-Chem Co., Ltd., Guangzhou, China), 10% astaxanthin in calcium lignosulfonate, was daily prepared by dissolving in warm water as recommended by manufacturer into dosages of 5 mg/kg astaxanthin for low dose administration and 100 mg/kg astaxanthin for high dose administration [20]. Mouse monoclonal anti-SOD1 antibody, HRP-conjugated mouse IgG kappa chain binding protein (m-IgGκ BP-HRP), goat polyclonal anti-GAPDH antibody and HRP-conjugated donkey anti-goat IgG antibody were purchased from Santa Cruz Biotechnology, Inc., Dallas, TX, USA. DAB peroxidase substrate kit was purchased from Vector Laboratories, Inc., Burlingame, CA, USA.

**Experimental design**
The experimental animals were randomly divided into 4 groups of 5 rats: Control group (normal rats without AFB\textsubscript{1} intoxication and AX treatment), AFB\textsubscript{1} group (rats intoxicated by AFB\textsubscript{1}), AXL+AFB\textsubscript{1} group (rats treated with low dose AX before AFB\textsubscript{1} intoxication) and AXH+AFB\textsubscript{1} group (rats treated with high dose AX before AFB\textsubscript{1} intoxication). The rats in control and AFB\textsubscript{1} groups were gavaged with 2 ml of distilled water for 7 days. At day 8, control rats were received 2 ml of 50\% DMSO in distilled water whereas rats in AFB\textsubscript{1} group were received 1 mg/kg AFB\textsubscript{1}. The rats in AXL+AFB\textsubscript{1} and AXH+AFB\textsubscript{1} groups were gavaged for 7 days with 5 mg/kg and 100 mg/kg astaxanthin, respectively. Then, both groups were also received 1 mg/kg AFB\textsubscript{1} at the 8\textsuperscript{th} day. All rats were fed for the following 4 days and were euthanized at the 13\textsuperscript{th} day by intraperitoneal injection with 60 mg/kg pentobarbital sodium and blood and liver tissue samples were then collected. During the experiment periods, the rats were weighed every day to observe weight gain.

**Serum biochemistry**

The blood samples were obtained from the experimental rats by cardiac puncture. The serum was separated and measured the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by automated chemistry analyzer (Mindray\textsuperscript{\textregistered} BS 380, Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

**Histopathological examination**

Entire liver was removed from each rat and weighed. The ratio of liver weight/100 g body weight (LW/100 g BW) was calculated. The liver tissue for histopathological study was cut and fixed in 10\% neutral buffered formalin, dehydrated in graded alcohol series, cleared in xylene and embedded in paraffin. Tissue sectioning was done at 3\textmu m thickness and stained with hematoxylin and eosin (H&E). The histopathological observation was performed by Olympus\textsuperscript{\textregistered} BX50 light microscope (Olympus Corporation, Tokyo, Japan). The photographs of 40x magnification were
taken and the cell numbers of hepatocyte alterations were counted for 20 randomized areas of a square millimeter (mm²).

**Immunohistochemical staining**

Paraffin sections of liver were deparaffinized, rehydrated and incubated in 10 mM citrate buffer (pH 6.0) at 95°C for antigen retrieval. Non-specific binding was blocked with 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Mouse monoclonal anti-SOD1 antibody at a dilution of 1:500 was overnight incubated on liver tissue at 4°C. The tissues were washed with 0.05% Tween 20 in PBS (pH 7.4) and incubated with 3% hydrogen peroxide to inactivate endogenous peroxidase. The m-IgGκ BP-HRP at a dilution of 1:1,000 was applied and immunoreactivity of SOD1 was visualized by DAB peroxidase substrate kit. The tissue sections were then counterstained with hematoxylin and observed under light microscope.

**Western blot analysis**

The liver tissue was snap frozen by liquid nitrogen and kept in -80°C refrigerator. Total protein was extracted from homogenized tissue in RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich). The supernatant was collected after centrifugation at 13,000 rpm for 20 min at 4°C. The protein concentrations of each sample were measured using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed by Synergy H1 Hybrid Multi-Mode Microplate Reader with Gen5™ 2.0 Data Analysis Software (BioTek®, Winooski, VT, USA). The samples containing 25 μg of total protein were separated by 12.5% SDS-PAGE (100V for 90 min) and then transferred into nitrocellulose membrane by constant voltage of 100V for 1 hr at 4°C. The non-specific protein binding on the membrane was blocked with 5% non-fat milk in TBST and the membrane was then incubated with mouse monoclonal anti-SOD1 antibody (1:2,000 dilution). After washing with TBST, the membrane was incubated with m-IgGκ BP-HRP (1:5,000 dilution). The bands were detected by enhanced
chemiluminescence (ECL) (Clarity™ Western ECL Substrate), visualized by ChemiDoc™ Imaging System and analyzed by Image Lab™ Software (version 6.0) (Bio-Rad Laboratories, Inc.). The same membrane was immersed in stripping buffer at 50°C for 30 min. The non-specific protein was blocked with 5% BSA and the membrane was reprobed with goat anti-GAPDH antibody (1:1,000 dilution) and HRP-conjugated anti-goat IgG antibody (1:5,000 dilution) as an internal control.

**Data analysis**

The average values of body weight gains, LW / 100 g BW, liver enzyme parameters, numbers of hepatocyte alterations and normalized densitometry of SOD1/GAPDH from western blot analysis were presented as mean ± standard deviation (SD). The percentage of SOD1 stained area was measured by Image J (version 1.52a, National Institute of Health, Bethesda, MD, USA) from 5 different areas in each group and averaged to evaluate the density of SOD1 immunoreactivity. All data were analyzed by using Kruskal-Wallis test followed by Dunn’s post hoc test. The R statistical software for Windows (version 3.5.0., R Core Team) was used to analyze statistical significance with an acceptable level at $p \leq 0.05$.

**RESULTS**

**Effect of AX and AFB\textsubscript{1} on body weight**

During the experimental period, none of the rats showed any signs of illness. All rats were daily weighed and their body condition was examined for health assessment. The variation of average body weight gain in rats of all groups either before or after AFB\textsubscript{1} intoxication showed no significance. Interestingly, the rats in AXH+AFB\textsubscript{1} group that have been gavaged by high dose AX for 7 days before AFB\textsubscript{1} administration gained less weight than others. However, the weight gain of rats in AFB\textsubscript{1} group became lowest after AFB\textsubscript{1} intoxication (Table 1).
Effect of AX and AFB\textsubscript{1} on liver weight (LW)

At 13\textsuperscript{th} day, all rats were weighed and then euthanized to collect liver samples. An average body weight of rats in all groups showed no significant difference. However, the LW was significantly increased in AFB\textsubscript{1} group, while it was not different among control, AXL+AFB\textsubscript{1} and AXH+AFB\textsubscript{1} groups. The calculation of \text{LW}/100 \text{ g BW} manifested significantly higher value in AFB\textsubscript{1} group when compared to control and both AX treated groups (Table 2).

Effects on liver function evaluated by serum ALT and AST levels

All experimental groups showed elevated serum ALT and AST as compared to control (Table 3). Especially, the level of serum ALT in AFB\textsubscript{1} group was significantly increased whereas this parameter revealed no significant difference from control in AXL+AFB\textsubscript{1} and AXH+AFB\textsubscript{1} groups. Nevertheless, average AST level of rats in control, AFB\textsubscript{1}, AXL+AFB\textsubscript{1} and AXH+AFB\textsubscript{1} groups was not statistically different.

Liver histopathological findings

The histopathological changes of liver tissue occurred in all experimental groups apart from control rats, which revealed normal pattern of hepatic cord arrangement in hepatic lobules with intact portal areas (Fig. 1a). Besides normal hepatocytes, some megalocytic, binucleated and apoptotic hepatocytes were also presented in control group. The apoptotic cells were smaller than adjacent cells and showed hypereosinophilic cytoplasm from cytoplasmic condensation with pyknotic or fragmented nuclei [13] (Fig. 1b). After receiving 1 mg/kg AFB\textsubscript{1} for 4 days, massive vacuolar degeneration of hepatocytes were found in all hepatic lobules of rats from AFB\textsubscript{1} group. Some hepatocytes underwent karyorrhexis and some degenerated cells exhibited really faint or nuclei absent (karyolysis) which indicated necrosis (Fig. 1c and 1d). Various degrees of typical bile duct hyperplasia and inflammatory cell infiltration were observed in all portal areas. Acidophilic megalocytes and binucleated hepatocytes
were distributed in all lobules, though some of them had been degenerated (Fig. 1d). The rats in AXL+AFB₁ group gavaged by 5 mg/kg AX for 7 days before AFB₁ intoxication revealed different results compare with the AFB₁ treatment group. The vacuolar degenerative hepatocytes were found at periportal area, but were extended into midzonal to centrilobular areas in some lobules (Fig. 1e). The amount of megalocytes, binucleated cells and inflammatory cell infiltration were approximately equal to AFB₁ group (Fig. 1f), whereas bile duct hyperplasia was low. Noteworthy, one rat from this group showed distinct appearance of hypertrophic hepatocytes with extensive basophilic cholangiocyte proliferation. The vacuolar degenerative hepatocytes were rarely noticed in this rat. These hypertrophic cells were approximately two times larger in size when compared with normal hepatocyte, leading to disarrangement of hepatic cord. The sinusoids in these areas were narrowed. Some cells showed abnormal large sized nucleus and some were binucleated, but most cells possessed increasing cellular content but normal nuclear sizes with irregular cytoplasmic clumping below the plasma membrane and vesicular pattern of nuclei (Fig. 2). In AXH+AFB₁ group, one rat showed hepatic vacuolar degeneration and typical bile duct hyperplasia at periportal and midzonal areas with less severity than in AXL+AFB₁ group (Fig. 1g and 1h) but the others revealed predominant hypertrophic hepatocytes and cholangiocyte proliferation. Among these four rats in AXH+AFB₁ group, half of them showed slight hepatic vacuolar degeneration, but this occurrence was not existed within any lobules in another half. The mean value of all hepatocyte alterations in control group was the lowest except the number of apoptotic cells which was insignificantly higher than in AFB₁ and AXL+AFB₁ group. The degeneration and necrosis were remarkably high in AFB₁ group compared to other groups (p<0.05). Moreover, the AXL+AFB₁ group also showed significantly notable level of hepatocyte degeneration from AXH+AFB₁ group. The number of megalocyte was higher in AFB₁ and AXL+AFB₁ group than in AXH+AFB₁ group (p<0.05) while
the binucleated cells in AFB1 group was lower than in AXL+AFB1 and AXH+AFB1
groups (p<0.05). The AXH+AFB1 group revealed low amount of necrotic cells which
was not significantly different from control but the level was elevated in AXL+AFB1
group (p<0.05). In contrast, the amount of apoptotic cells was high in AXH+AFB1 group
implying that the irreversibly degenerative cells in AXH+AFB1 group were subjected to
be eliminated by apoptosis instead of necrosis. The highest level of hypertrophic
hepatocytes was also apparently observed in AXH+AFB1 group though there was no
significant difference among groups (Table 4).

**Immunohistochemistry and expression of SOD1**

The SOD1 immunostaining of liver cells in control group revealed of dark brown
color either in cytoplasm or nuclei (Fig. 3a) whereas degenerative cells in AFB1 group
were stained only at nuclei and edge of cells and the necrotic cells were unstained. Very
few cells were fully stained in the cytoplasm (Fig. 3b). For AXL+AFB1 group, the
hepatocytes displayed variety of immunoreactivity but mostly were illustrated as moderate
brown staining (Fig. 3c). The higher immunostaining was seen in hepatocytes of
AXH+AFB1 group, which were filled by visible dark brown color (Fig. 3d). The
percentage of brown staining area from each group was quantified to indicate differences
of SOD1 expression level. The SOD1 immunostaining from control, AFB1, AXL+AFB1
and AXH+AFB1 groups was shown as 37.61%, 31.47%, 33.16% and 35.43%,
respectively. The analysis of these measurement implied that SOD1 expression in
hepatocytes of AXH+AFB1 group was significantly elevated comparing to AFB1 and
AXL+AFB1 groups. Control group significantly presented the highest level of SOD1
immunostaining whereas the percentage of staining area between AFB1 and AXL+AFB1
groups was not significantly different (Fig. 4). The western blot analysis was performed
in order to confirm whether AX could induce expression of SOD1 in AFB1 ingested rat
as shown in immunohistochemistry. The result manifested that SOD1 expression was
slightly increased in AXH+AFB_1 group from the control but markedly declined in AFB_1 group (p<0.05). However, the expression level of SOD1 was not much different between AFB_1 and AXL+AFB_1 groups (Fig. 5).

**DISCUSSION**

Although the body weight gain in all groups was not significantly different during the first week of experimental period, the declined weigh and food intake of rats in AXH+AFB_1 group has been noticed. No sign of illness was examined in these rats. Since the AX used in this experiment is a feed additive grade for aquatic animals, it was 10% mixture with calcium lignosulfonate to facilitate the administration into water-based foods. We speculate that highly viscous ingredients might completely fill in rat stomach and alleviate appetite. Indeed, an overdose of vitamin A has been known to induce appetite loss and growth reduction [24], but an AX dosage of 100 mg/kg has been orally administered in rats for 15 days without any adverse effect [20]. Therefore, the dosage used in this study might not high enough to promote toxicity. After AFB_1 gavage, the difference of body weight gain between groups was also insignificant. Nevertheless, an average weight gain of rats from AFB_1 group was obviously less than others, whereas the AXH+AFB_1 group was elevated. Qian et al. (2013) showed similar result in the toxicological study of AFB_1 exposure in rats, but the body weight gain of 1 mg/kg AFB_1 exposed rats was significantly decreased when compared to the control [34]. This result implies that AX could help to enhance body weight in rats receiving AFB_1.

An average LW and LW / 100 g BW of rats in AFB_1 group was significantly higher than other groups, while those of rats in control, AXL+AFB_1 and AXH+AFB_1 groups showed little difference. From our study, the acute intoxication of high dose AFB_1 recruited inflammatory response in liver tissue which was shown as inflammatory
cells infiltration around portal areas. This occurrence was also consistent with reports of some studies indicating that an increased liver weight was subsequently a result from massive liver inflammation [20, 34]. Noteworthy, the insignificant difference of average LW and LW / 100 g BW between control and both of AX treated groups implies that AX possessed an anti-inflammatory potential in rat liver towards acute AFB₁ intoxication. Previous studies revealed that AX reduces pro-inflammatory cytokines which cause liver damage by a toxic dose of acetaminophen, such as TNF-α and IL-6, within 24 hr [50]. Beside the inhibition of acute inflammation, AX also inhibits chronic inflammation which could induce hepatic fibrosis by decreasing of TGF-β1 expression via down regulation of NF-κB [22, 39].

AX could restore liver function from AFB₁ induced destruction as inspected by the significantly declining level of ALT in rats received AX for 1 week before AFB₁ intoxication (Table 3). The AST level, despite no statistically significant difference among groups, the level in AXH+AFB₁ group was only slightly different from AFB₁ group, whereas this enzyme in AXL+AFB₁ group had almost similar level to control group. It is surprising that an equal dosage of AX used significantly reduced serum ALT and AST levels in CCl₄ induced rats [20], although we gave half shorter duration of AX treatment before inducing hepatocellular damage by AFB₁. We also suspect whether calcium lignosulfonate might have hepatotoxic action since its high concentration has been mixed with AX and gavaged into rats in this group. However, this hypothesis might be untrue because this polymer of lignin is considered safe as a feed additive in animals [12]. As a result of its high molecular weight, calcium lignosulfonate is poorly absorbed from the gastrointestinal tract of rats. In addition, the in vitro studies also show low transepithelial transport in Caco2 cells. Therefore, the presence of substantial amount of this compound in the tissues is unlikely [11]. Furthermore, the JECFA set an acceptable
daily intake of calcium lignosulfonate, based on a 90-day rat study, at a dosage up to 2,000 mg/kg body weight [19, 42]. Accordingly, such amount of calcium lignosulfonate given to rats in AXH+AFB₁ group is not possible to cause hepatotoxic. Moreover, it was also confirmed that the causation of the lowest weight gain of rats in AXH+AFB₁ group in the first period of our experiment was from an indigestible calcium lignosulfonate as described above.

The AFB₁ is known to have hepatotoxic potency. In this study, massive degeneration, necrosis, magalocytosis, and binucleation of hepatocytes were induced in the liver of rats administered a high single dose of AFB₁ (Fig. 1c and 1d). These incidences were found throughout the hepatic lobules but mostly localized at periportal to midzonal areas. The finding is similar to a report of Qian et al. (2013) [34] but contrast to the chronic intoxication by repeating low dosage of AFB₁ which hydropic degeneration was primarily detected at centrilobular (periacinar) and midzonal regions of hepatic lobules [48]. The hepatocytes residing around centrilobular area own the highest concentration of microsomal cytochrome P450 which activates AFB₁ metabolism to produce a highly reactive intermediate, the 8, 9-epoxide metabolite [10, 41]. Therefore, the centrilobular hepatocytes tend to be more sensitive to AFB₁ induced liver damage than the periportal ones. Nevertheless, the AFB₁ from intestinal absorption is circulated in portal vein located in portal area before distributing toward the centrilobular region. We speculate that, after oral administration with high dose AFB₁, the amount of AFB₁ predominantly concentrated and metabolized in periportal and midzonal hepatocytes was high enough to immediately degenerate and destroy cells. Thus, the vacuolar degeneration and necrosis of hepatocytes were mainly focused in these areas instead of centrilobular region. The ROS produced from AFB₁ metabolism affect cell membrane integrity leading to calcium influx and cell swelling as the initial process of vacuolar degeneration [8]. This pathological change was declined in
AXL+AFB\textsubscript{1} group (Fig. 1e and 1f) and obviously reduced in AXH+AFB\textsubscript{1} group (Fig. 1g and 1h) indicating the protective effects of AX to the cell from degenerative process. The damage of cell membrane is primarily activated by peroxyl radical generated in the process of lipid peroxidation, and carotenoids are known the efficient scavenger for this ROS [31]. Therefore, AX should certainly have scavenging efficacy against peroxyl radical as in other carotenoids. Besides cellular degeneration and necrosis, megalocytosis and binucleation of hepatocytes were also noted in all groups especially in AFB\textsubscript{1} administered groups. Since aflatoxin has antimitotic effect, though less capable than pyrrolizidine alkaloid, the replacement of AFB\textsubscript{1} damaged hepatocytes by naturally proliferation is limited, especially in the prolonged aflatoxicosis [10, 30]. As a result, the cellular and nuclear enlargement of cell displaying active DNA and protein synthesis but impaired cell division is occurred, so called megalocytosis [3]. However, the feasibility of megalocyte as a precancerous cell is not confirmed although the study of liver carcinogenesis in Wistar rats with a single dose of 7 mg/kg AFB\textsubscript{1} or subsequently repeated CCl\textsubscript{4} inhalation demonstrates that megalocytosis is the first observed lesion and the tumor cells usually located among the megalocytic cells [38]. In another aspect, polyploidy, which is usually found as megalocyte and binucleated cell, could also denote an adaptive mechanism to become more resistant to oxidative stress and genotoxic damage from xenobiotic [4, 14, 17, 28]. Several researchers elucidated that diploid hepatocytes enter the cell cycle faster than polyploid cells. Moreover, multiple copies of tumor suppressors in polyploid hepatocytes reduce the likelihood of transformation, whereas diploid hepatocytes have only two copies and are prone to tumorigenesis by inactivating mutation of these genes. These advantages of polyploidy eventually reduce the expansion of transformed genes into the progeny [14, 46]. According to our result, we hypothesized that increased megalocyte in AFB\textsubscript{1} ingested rat was due to dual causation, AFB\textsubscript{1} toxicity and adaptation to toxin. AX treated groups exhibited less
number of megalocyte than in AFB$_1$ group because it could alleviate AFB$_1$ toxicity. Meanwhile, the adaptive response in AX treated groups shown as polyploidy led to higher number of binucleated hepatocyte (Table 4).

The bile duct hyperplasia exhibited in AFB$_1$ group was a typical proliferation of cholangiocytes which demonstrated as newly formed bile ducts with well-defined lumens [23]. This is relevant to an observation from Qian et al. (2013) in rats orally administered by 1 mg/kg AFB$_1$ [34]. In AXL+AFB$_1$ group, most rats also showed similar morphology of bile duct hyperplasia, except one rat that revealed cholangiocyte proliferation originated at portal area and extended into surrounding hepatic parenchyma coincided with hypertrophy of hepatocytes (Fig. 2). The same incidence was also noted in most rats from AXH+AFB$_1$ group. This atypical cholangiocyte proliferation was supposed to be a ductular reaction to restore the nearby damaged hepatocytes during liver injury. The regeneration is arisen from transdifferentiation of proliferated cholangiocytes into hepatocytes [37]. In acute liver injury, destroyed hepatocytes are replaced by $\beta$-catenin dependent proliferation of neighboring alive hepatocytes. However, the $\beta$-catenin dependent regenerative response is impaired in case of severe or chronic injury. Thus, the hepatocyte repopulation will be relied on $\beta$-catenin independent cholangiocyte-to-hepatocyte differentiation that will be occurred after damaging causes have been eradicated [32]. AX is well documented for its anti-oxidative activity. The elimination of free radicals and ROS generated from AFB$_1$ metabolism is considered to remove cause of liver damage, leading to improvement of liver tissue by atypical cholangiocytes proliferation, as seen in some rats from AXL+AFB$_1$ and AXH+AFB$_1$ groups. Ultimately, these cells will be derived into hepatocytes. Moreover, the hepatocytes predominantly found in these rats showed the morphology of hypertrophy with cytoplasmic clumping and vesicular nucleus.
According to a review of Hall et al. (2012), an increased size of liver cell could be resulted from elevated protein production and proliferation of subcellular organelles typically sER and/or peroxisome. From the experiment of microsomal enzymes inducer administration in rats, the hypertrophy of hepatocytes was examined through smooth ER proliferation observed by electron microscopic study which demonstrated characteristic stacks of smooth ER that crowd out other organelles. Some xenobiotics also induce peroxisome proliferation which presented as catalase positive electron dense vesicles in the cytoplasm. Importantly, both circumstances are considered adaptive and non-adverse responses to induce hepatic metabolism. However, these responses can be failed at higher dose levels or following prolonged exposure, if degenerative changes of hepatocytes are overcome or novel cytotoxic metabolites are generated [16]. These data supports our observations, especially in AXH+AFB\textsubscript{1} group, revealing hepatocyte hypertrophy which was suspected to contain cytoplasmic proteins with accumulation of multiplied organelles involving production of those anti-oxidative enzymes (Table 4). Moreover, the higher serum ALT level, but not above two times than control, in AXH+AFB\textsubscript{1} group might indicate a consequence of increased enzyme synthesis, as also noted by Hall et al. (2012) [16].

In the liver, the AFB\textsubscript{1} toxicity through AFB\textsubscript{1}-8, 9-epoxide causes generation of intracellular ROS leading to oxidative stress and damage of vital components such as DNA and cell membrane [21, 25, 27]. The cellular anti-oxidative activities are deprived in 1 mg/kg AFB\textsubscript{1} administered rats as shown by increasing lipid peroxidation and decreased various anti-oxidative enzymes, such as SOD, CAT, GPX and glutathione-S-transferase [33]. SOD has been shown to have a protective effect on liver injury induced by AFB\textsubscript{1} [20, 27, 40, 48, 49]. It is responsible for the first line of antioxidant defensive system against cell injury from the highly ROS generated by biochemical redox reactions in normal cell metabolism, and by exogenous sources. The SOD\textsubscript{1} expression is
stronger in normal liver tissue comparing to hepatocellular carcinoma in the same patient. However, superoxide radicals and other ROS generated in malignant cells are probably diffuse into surrounding tissue and further damaging it. The up-regulation of SOD and other antioxidative enzymes is likely the mechanism to resist the damage from increasing oxidative stress in hepatocyte [45]. SOD1 is ascribed to be synthesized by ribosomes. Its distribution in the cytosol is unsuspicious but the existence in nuclear matrix is believed to occur by diffusion of cytosolic SOD1 into the nucleus through nuclear pores [6]. The localization of SOD1 is corresponding to the finding from our present study. Additionally, our study obviously revealed the SOD1 expression decreased in the liver of rats in AFB\(_1\) group, whereas the expression of this enzyme in AXH + AFB\(_1\) group was significantly higher (Figs. 4 and 5). This result implies that AX potentially induces SOD1 expression as same as in previous reports [5, 20]. The similar result has been demonstrated in a study of curcumin hepatoprotective effects against aflatoxicosis. It was also found that curcumin stimulates up-regulation of anti-oxidative enzyme gene expression such as SOD and GPX. Additionally, curcumin also demonstrates other anti-oxidative activities by scavenging ROS through interaction within the oxidative cascade and inhibiting aflatoxin biotransformation by restriction of cytochrome P450, thus decreasing the formation of AFB\(_1\)-8, 9-epoxide [27]. Though we did not intensively study for anti-oxidative effects of AX as in curcumin, the existing results are substantial to conclude that AX possesses hepatoprotective role in rat aflatoxicosis. The AFB\(_1\) generated ROS that consequently damaged hepatocytes as shown by elevated liver enzyme parameters and histopathological changes of vacuolar degeneration and cell necrosis. AX could alleviate cellular damage by diminishing degenerative and necrotic changes as well as serum ALT level in AXL+AFB\(_1\) and AXH+AFB\(_1\) groups. Some rats from AX treated groups also exhibited atypical proliferation of cholangiocytes which is believed to be a process of transdifferentiation
to replace damaged hepatocytes. Likewise, both AX treated groups demonstrated higher percentage of SOD1 stained area when compared to AFB₁ group. This result also corresponds to the western blot analysis indicating that SOD1 expression was induced by AX. However, the values in AXL+AFB₁ group were not significantly different from AFB₁ group, denoting that the AX induced SOD1 expression was dose dependent. The level of SOD1 expression in AXH+AFB₁ group that was above the control could also verify increased activity of SOD1 production. Additionally, the hypertrophy of hepatocytes mostly found in rats receiving 100 mg/kg AX prior to AFB₁ administration in AXH+AFB₁ group may imply the over production of detoxifying proteins and accumulation of involving cytoplasmic organelles to mitigate AFB₁ toxicity.

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FIGURE LEGENDS

Figure 1 The illustration of histopathological findings of rat liver in control group (a) showed normal histology which some megalocytes, binucleated hepatocytes and apoptotic cells could be noticed (b). Liver tissue from AFB1 group (c) revealed massive vacuolar degeneration in all lobules and bile duct hyperplasia in portal area. Megalocytes, binucleated hepatocytes and their degenerated forms as well as necrotic cells that underwent karyorrhexis were extensively found in this group (d). The vacuolar degeneration of hepatocytes was gradually reduced from AXL+AFB1 group to AXH+AFB1 group (e and g). The appearance of megalocytes and binucleated cells
was still high in AXL+AFB<sub>1</sub> group (f). Necrotic and apoptotic cells were slightly observed in AXL+AFB<sub>1</sub> and AXH+AFB<sub>1</sub> groups (h).

**Note:** Thick arrow; Megalocyte, Black arrowhead; Binucleated hepatocyte, Black thin arrow; Degenerative binucleated hepatocyte, White thin arrow; Necrotic cell (karyorrhexis), White arrowhead; Apoptotic cell. AFB<sub>1</sub>: aflatoxin B<sub>1</sub>, AXL: low dose astaxanthin, AXH: high dose astaxanthin. H&E. Bar in a, c, e, g = 200μm, Bar in b, d, f, h = 50μm.

**Figure 2** Hypertrophic hepatocytes associated with proliferation of cholangiocytes (thin arrow) revealed in some rats of AXL+AFB<sub>1</sub> and AXH+AFB<sub>1</sub> groups. Few hepatocytes showed abnormal large sized nucleus (megalocytes; thick arrow) but most cells possessed increasing cellular content but normal nuclear size. H&E. Bar = 50μm.

**Figure 3** SOD1 Immunoreactivity demonstrated an expression in hepatocytes of (a) control, (b) AFB<sub>1</sub>, (c) AXL+AFB<sub>1</sub> and (d) AXH+AFB<sub>1</sub> groups. The other types of cell, such as Kupffer cells and endothelial cells, were stained blue from hematoxylin dye. Bar = 50μm.

**Figure 4** Percentage of SOD1 immunostained area from control, AFB<sub>1</sub>, AXL+AFB<sub>1</sub> and AXH+AFB<sub>1</sub> groups. AFB<sub>1</sub>: aflatoxin B<sub>1</sub>, AXL: low dose astaxanthin, AXH: high dose astaxanthin, SOD1: superoxide dismutase 1 (Cu/Zn-SOD).

**Figure 5** Western blot analysis of liver extract containing 25 μg of protein in each lane to detect SOD1 and GAPDH band density. Normalized densitometric values of SOD1/GAPDH, presented as mean ± SD, indicated that the SOD1 expression level in AXH+AFB<sub>1</sub> group was significantly higher than AFB<sub>1</sub> group (p<0.05). AFB<sub>1</sub>: aflatoxin B<sub>1</sub>, AXL: low dose astaxanthin, AXH: high dose astaxanthin, SOD1: superoxide dismutase 1 (Cu/Zn-SOD), GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.
Figure 1
Figure 2

Figure 3
Table 1: Average body weight gain before and after AFB₁ intoxication

| GROUP         | Body weight gain (g) |                |                |
|---------------|----------------------|----------------|----------------|
|               | Before AFB₁ (d1-d7)  | After AFB₁ (d9-d13) |
| Control       | 50±7.07              | 22±11.51       |
| AFB₁          | 38.75±2.50           | 13.75±4.79     |
| AXL+AFB₁      | 37±8.37              | 18.33±10.41    |
| AXH+AFB₁      | 33.33±11.55          | 20±14.14       |

Data is presented as mean ± SD. AFB₁: aflatoxin B₁, AXL: low dose astaxanthin, AXH: high dose astaxanthin.
Table 2  Average liver weight (LW), body weight (BW) and LW/100 g BW

| GROUP      | LW (g)       | BW (g)       | LW/100g BW |
|------------|--------------|--------------|------------|
| Control    | 9.71±0.14 a  | 242.50±13.23 | 4±0.24 a   |
| AFB1       | 11.50±0.54 b | 241.25±20.97 | 4.83±0.26 b|
| AXL+AFB1   | 9.72±0.70 a  | 225±9.13     | 4.33±0.28 a|
| AXH+AFB1   | 9.74±0.87 a  | 226.67±15.28 | 4.27±0.12 a|

Data is presented as mean ± SD. a, b indicate significant difference within a column (p < 0.05).
AFB1: aflatoxin B1, AXL: low dose astaxanthin, AXH: high dose astaxanthin.

Table 3  Serum ALT and AST levels of experimental rats in each group

| GROUP      | ALT (U/L)  | AST (U/L)  |
|------------|------------|------------|
| Control    | 24±9.08 a  | 68.80±29.23|
| AFB1       | 57.67±26.10 b | 122.33±33.50|
| AXL+AFB1   | 25.67±7.64 a | 78±28.69   |
| AXH+AFB1   | 42±9.90 a  | 143.50±54.45|

Data is presented as mean ± SD. a, b indicates significant difference within a column (p ≤ 0.05).
AFB1: aflatoxin B1, AXL: low dose astaxanthin, AXH: high dose astaxanthin, ALT: alanine aminotransferase, AST: aspartate aminotransferase.

Table 4  Effects of AFB1 and astaxanthin on hepatocyte alterations

| GROUP      | Average cell number per square millimeter (cells/mm²) |
|------------|------------------------------------------------------|
|            | vacuolar degenerative | megalocytic | binucleated | necrotic | apoptotic | hypertrophic |
| Control    | 12.3±15.9 a           | 7.9±10.8 a  | 44.6±30.6 a | 2.0±5.3 a | 2.5±5.0   | 0.0±0.0      |
| AFB1       | 747.8±229.7 b         | 54.6±54.5 b | 77.6±31.2 b | 117.8±62.0 b | 0.9±3.3 | 0.9±3.3      |
| AXL+AFB1   | 488.4±330.4 c         | 48.5±31.4 b | 93.9±39.2 c | 42.2±35.6 c | 1.8±7.2 | 2.0±7.2      |
| AXH+AFB1   | 47.7±42.9 d           | 26.2±19.8 c | 91.8±36.9 c | 5.2±7.7 a  | 3.2±6.2 | 32.5±26.8    |

Data is presented as mean ± SD. a, b, c, d indicates significant difference within a column (p < 0.05). AFB1: aflatoxin B1, AXL: low dose astaxanthin, AXH: high dose astaxanthin.
Effects of astaxanthin on axonal regeneration via cAMP/PKA signaling pathway in mice with focal cerebral infarction

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Abstract. – OBJECTIVE: To investigate the effect of astaxanthin on the neurological function of the middle cerebral artery occlusion (MCAO) mice and its possible mechanism.

MATERIALS AND METHODS: The male C57BL/6 mice were selected to establish the model of MCAO via electrocoagulation, and they were randomly divided into 4 groups: the sham operation group (Sham group), the cerebral ischemia model group (MCAO group), the astaxanthin intervention group (gavage with 30 mg/kg astaxanthin for 28 days, twice a day; Ast group), and astaxanthin + H89 group (Ast + H89 group). At 3, 7, 14, and 28 d after the operation, the Rotarod test and the balance beam footstep error test were performed. The brain tissues were taken for immunofluorescence to observe the expression of the growth-associated protein 43 (GAP43) in the cortex around the infarction. The GAP43 protein and mRNA levels in the cortex around the infarction were detected via Western blotting, and the Reverse Transcription-Polymerase Chain Reaction (RT-PCR), the levels of cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) in the bilateral cerebral cortex were detected via enzyme-linked immunosorbent assay (ELISA), and the PKAc and phosphorylated-cAMP-response element-binding protein (p-CREB) levels in the bilateral cerebral cortex were detected via Western blotting.

Biotin dextran amine (BDA) was injected at 14 d after the operation, and the brain was taken at 28 d. The BDA-labeled neurons or axons were observed in the bilateral cortex via immunohistochemistry and immunofluorescence, and the colocalization of BDA and GAP43 in the cortex around the infarction was observed using double immunofluorescence staining.

RESULTS: Compared with those in the MCAO group, the mean residence time in the Rotarod test was significantly increased, and the times of the footstep error on the balance beam were significantly reduced in the Ast group. In the Ast group, the expression of GAP43 in the cortex around the infarction, the GAP43 protein, and the mRNA levels were all significantly elevated. Immunofluorescence showed that in the Ast group, the number of the labeled neurons and axons in the bilateral cortex was slightly larger than that in the other groups, and the number of labeled axonal fibers in the ischemic cortex was significantly increased. The colocalization area of BDA and GAP43 was observed, and it was found that the positive area in the Ast group was significantly larger than that in the MCAO group. The cAMP level was higher in the Ast group and Ast + H89 group at 7, 14, and 28 d after operation, while the PKA level was lower in the Ast + H89 group at 7 and 14 d after operation and higher in the Ast group at 7, 14, and 28 d after operation. The results of the Western blotting manifested that the PKAc and p-CREB levels were upregulated in the Ast group at 7, 14, and 28 d after the operation, and downregulated in the Ast + H89 group at 7, 14, and 28 d after the operation.

CONCLUSIONS: Astaxanthin activates the cAMP/PKA/CREB signaling pathway by increasing the cAMP concentration in brain tissues, ultimately promoting the axonal regeneration in the cerebral cortex and improving the motor function.

Key Words: Astaxanthin, Cerebral Infarction, Axonal Regeneration, CAMP/PKA.

Introduction

Stroke is one of the most serious diseases threatening human health currently, whose mortality rate ranks first in all the diseases in China¹. Ischemic stroke is characterized by high morbidity, mortality, disability, and recurrence rates².
The health and quality of life of stroke patients survived, especially disabled patients, are seriously affected. Studies\textsuperscript{3-5} have found that after ischemic stroke, the neurological function recovers to a certain degree, indicating that the brain tissues have certain self-repairing capability. It has been found in the animal experiments that the intervention measures, such as appropriate drugs or exercise after cerebral infarction can stimulate endogenous axonal regeneration in brain tissues, which can benefit the reconnection of neural network and compensate for neurological functions of some denervated regions\textsuperscript{6}.

There are various influencing factors for axonal regeneration, and many complex intracellular and extracellular signal transduction mechanisms are involved. Currently, the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway is considered to be an important pathway affecting axonal regeneration\textsuperscript{7,8}. The cAMP keeps the vigorous neuronal growth by activating the PKA-mediated signaling pathway and can relieve the damage of the neuronal growth inhibitory factor to axonal growth cone by affecting the molecular effect caused by the downstream gene transcription, thereby promoting the axonal regeneration\textsuperscript{9,10}.

Astaxanthin, widely distributed in nature, is the pigment of crustaceans, which possesses the broad-pharmacological activities, and its neuroprotective effect has attracted much attention of the researchers\textsuperscript{11,12}. According to pharmacokinetic study, astaxanthin can be localized on the surface of the lipid membrane or passes through the lipid membrane, and it can also pass through the blood-brain barrier of rodents, thus achieving a better efficacy on the nervous system diseases. Some reports\textsuperscript{13,14} have found that astaxanthin can significantly inhibit the expression of interleukin-1α (IL-1α), IL-6, and tumor necrosis factor-α (TNF-α) in the brain and improve the lipopolysaccharide-induced neuroinflammatory response in the brain of mice. Moreover, astaxanthin, through simulating the neurotrophic factors and promoting synaptic survival, can alleviate the cortical damage volume, neuronal loss, and neural degeneration\textsuperscript{15,16}. However, there are few investigations on the effect of astaxanthin on cerebral infarction. In this study, C57BL/6 mice were used as an object of study, the focal middle cerebral artery occlusion (fMCAO) model was established via electrocoagulation, the restorative effect of astaxanthin on the neurological function of MCAO mice was observed, and its possible mechanism and signaling pathway were investigated, so as to provide new ideas for the mechanisms of cerebral infarction and nerve repair.

**Materials and Methods**

**Laboratory Animals and Models**

A total of 100 male C57BL/6 mice aged 8-12 weeks old weighing 25-30 g were purchased from the Shanghai SLAC Laboratory Animal Company (Shanghai, China). The mouse model of MCAO was established \textit{via} electrocoagulation\textsuperscript{17}: the mice were fasted for solids and liquids before the operation, and they were anesthetized \textit{via} intraperitoneal injection of tribromoethanol (0.4 g/kg). After successful anesthesia, the mice were fixed in a supine position, a median incision was made on the neck, and the right common carotid artery (CCA) was exposed, separated, and permanently ligated. Then, the mice were fixed in a left lateral position, an incision was made along the line between external auditory canal and medial canthus, the skin was separated, the temporalis muscle was fixed on the right under a stereoscopic microscope, and the MCA was positioned under the skull. The skull was worn using the dental drill right above the MCA until the vessels were exposed, and the MCA was carefully burned using the single-pole electrocoagulator till coagulation. In the Sham operation group, the operations were the same as those in the operation group, but the CCA was not ligated, and the MCA was not coagulated. This study was approved by the Animal Ethics Committee of Tengzhou Central People’s Hospital.

**Animal Grouping and Drug Administration**

The mice were randomly divided into 4 groups: the sham operation group (Sham group, \(n=20\)), cerebral ischemia model group (MCAO group, \(n=20\)), astaxanthin intervention group (Ast group, \(n=20\)), and astaxanthin + H89 group (Ast + H89 group, \(n=20\)). Astaxanthin was prepared with olive oil for postoperative gavage (30 mg/kg), twice a day for 28 d. H89, a PKA inhibitor, was dissolved in ultrapure water (4 μg/μL) and injected into the ventricle (2 μL) using the stereotaxic apparatus before modeling. Biotin dextran amine (BDA) was injected into the cortex at 14 d after the operation as follows: the mice were anesthe-
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tized with 10% chloral hydrate and fixed on the stereotaxic apparatus in a prone position. Then, the anterior fontanel was exposed, and BDA was injected into the left motor-sensory cortex in two points (1 μL/point).

**Rotarod Test**

The Rotarod test was performed at 3, 7, 14, and 28 d after the operation. The mice were placed on the rotarod rotating at 4 rpm in a quiet environment, the speed was gradually increased from 4 rpm to 40 rpm, and the total test time was not more than 300 s. The test was terminated when the mice fell off the rotarod. The test was repeated for 3 times, and an adaptive training was given for mice at 1 d before modeling.

**Balance Beam Footstep Error Test**

The test was performed at 3, 7, 14, and 28 d after the operation. The mice were placed on a balance beam (L×W×H: 120 cm × 0.6 cm × 60 cm) with a platform set at one end. The mice walked through the balance beam, and they usually turned 180°C and continued to walk to the other end once reaching the end of the balance beam. If the left hind limb or forelimb slid down from the balance beam, the footstep error was recorded once. The total times of footstep errors within 50 steps were recorded. The test was repeated 3 times, and an adaptive training was needed for mice before modeling.

**Immunofluorescence Staining**

At 7, 14, and 28 d after the operation, the mice were anesthetized with chloral hydrate and infused with 4% paraformaldehyde into the heart. The brain was taken, and the brain tissues were stored in 4% paraformaldehyde solution at 4°C for 48 h, and then placed in 30% sucrose solution until they completely sank to the bottom. Then, the brain tissues were taken, sliced into 30 μm-thick sections using a freezing microtome, sealed with 10% donkey serum at room temperature for 1 h, and incubated with the primary antibody on a shaking table at 4°C overnight. After that, the sections were re-warmed for 1 h and incubated with the secondary antibody. After the anti-fluorescence attenuating agent was added, the sections were sealed, and the images were acquired under a fluorescence microscope.

**Western Blotting**

At 7, 14, and 28 d after the operation, the mice were decollated, and the brain was taken. The total protein was extracted according to the total protein extraction kit (Beyotime, Shanghai, China), and the protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Abcam, Cambridge, MA, USA). The discontinuous sodium dodecyl sulfate-polyacrylamide gel was prepared, and 50 μg total proteins in each group were loaded into the loading well for electrophoresis. Then, the protein was transferred onto a polyvinylidene difluoride (PVDF) membrane, sealed with 5% skim milk for 1 h, and incubated with the primary antibody diluted with Tris-Buffered Saline and Tween-20 (TBST) on a shaking table at 4°C overnight. After the protein was re-warmed on the next day, it was incubated again with the secondary antibody at room temperature for 2 h. After chemiluminescence image development, the protein was scanned using the far-infrared fluorescence scanning imaging system.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**

The total RNA was extracted from 100 mg brain tissues on the infarct side, and the purity and content of RNA were detected. After the total RNA was reversely transcribed at 42°C for 50 min, and the reverse transcriptase was inactivated at 95°C for 5 min, the PCR amplification was performed. GAP43: Forward: 5’-AGAAGGAGGGAGATGGCT-3’, Reverse: 5’-CTTGGGAGGACGGGGAGTT-3’. glyceraldehyde 3-phosphate dehydrogenase (GAPDH): Forward: 5’-CCTTCCGTGTTTCTACCC-3’, Reverse: 5’-CCCAAGATGCCCTTCAGT-3’. After amplification, the data were quantitatively analyzed using the 2-ΔΔCT method with GAPDH as an internal reference gene.

**Diaminobenzidine (DAB) Immunohistochemistry**

DAB was injected into the mice at 14 d after the operation, the mice were decollated, and the brain was taken at 28 d. Then, the brain was fixed and sliced into sections in the same way as above. The sections were incubated with the avidin-HRP (Horse Reddish Peroxidase) working solution for 4 h, followed by color development with DAB working solution for 15 min, dehydration with gradient alcohol, transparentization with xylene, and sealing. Finally, the images were acquired under an optical microscope.
Enzyme-Linked Immunosorbent Assay (ELISA)

The mice were decollated, and the brain was taken at 3, 7, 14, and 28 d after the operation. The brain was immediately weighed and homogenized in a pre-cooled 2 mL homogenate tube. The homogenate was collected and centrifuged using the low-temperature ultra-speed centrifuge at 4°C and 2000 rpm for 15 min, and the supernatant was taken. The concentrations of cAMP and PKA were measured according to the instructions of the ELISA kit (R&D Systems, Minneapolis, MN, USA) taken from a refrigerator at 4°C.

Statistical Analysis

The Statistical Product and Service Solutions (SPSS) 19.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analysis. The detection results were expressed as Mean ± SEM (Standard Error of Mean). The analysis of variance (ANOVA) was performed for the data comparison among groups, and the homogeneity test of variance was used. In the case of a significant difference in ANOVA, the Student-Newman-Keuls (SNK) test was further used for pairwise comparison. The nonparametric rank test was adopted in the case of heterogeneity of variance. *p<0.05 suggested that the difference was statistically significant.

Results

Astaxanthin Promoted the Recovery of Motor Function of Mice After Ischemic Infarction

It was found in the Rotarod test that the mean residence time in the Sham group was longer than 300 s at each time point after the operation. Compared with that in the MCAO group, the mean residence time of mice in the Ast group was markedly increased at 7, 14, and 28 d after the operation. The mean residence time of mice in the Ast + H89 group was shorter than that in the MCAO group at 14 and 28 d after the operation, and there were statistically significant differences (Figure 1A). The results of the balance beam footstep error test revealed that there were almost no footstep errors at each time point after the operation in the Sham group. The times of footstep errors were fewer in the Ast group than that in MCAO group at 7, 14, and 28 d after the operation, showing statistically significant differences, and it was larger in the Ast + H89 group than that in the MCAO group at 7, 14, and 28 d after operation (Figure 1B).

Astaxanthin Stimulated the Axonal Regeneration of Cortex Around the Infarction After Ischemic Infarction

In terms of GAP43 fluorescence expression in the cortex around the infarction, the GAP43 expression was significantly increased in the MCAO group compared with that in the Sham group at 7, 14, and 28 d after the operation, and it was further increased in the Ast group compared with that in the MCAO group, displaying a statistically significant difference. The GAP43 expression in the Ast + H89 group significantly declined compared with that in the Ast group (Figure 2A). The GAP43 protein and mRNA expressions in the cortex around the infarction showed consistent trends. At 7, 14, and 28 d after operation, the GAP43 protein and mRNA expressions were always lower in the Sham group than those in the MCAO group, they were overtly increased in the

Figure 1. Astaxanthin promoted the recovery of motor function of mice after ischemic infarction. A, Analysis of average ridding time in different groups. B, Analysis of average times of footstep error in different groups. *p<0.05 vs. MCAO group.
Astaxanthin promotes axonal regeneration

Astaxanthin promotes axonal regeneration

Astaxanthin Promoted the Reconnection of New Axons of Cortex Around the Infarction

According to immunohistochemistry, the neurons and new axons taking in BDA were labeled brown yellow. In each group, there were a certain number of labeled neurons and their axons in the cortex on the unaffected side, as well as a certain number of axonal fibers in the cortex around the infarction without labeled neurons. There were very few BDA-labeled neurons and axons in the Sham group (Figure 3A). The immunofluorescence staining revealed that the neurons and new axons taking in BDA were labeled red. It was found in the semi-quantitative analysis that the number of labeled neurons and axons in the contralateral cortex in the Ast group was slightly larger than that in the other groups, and there were no significant differences among groups except for the Sham group. Compared with that in the MCAO group, the labeled axonal fibers in the cortex on the ischemic side was remarkably increased in the Ast group, and declined in the Ast + H89 group, displaying statistically significant differences (Figure 3B). The colocalization area of BDA (red) and GAP43 (green) was observed in the cortex around the infarction, and it was found that the positive area in the MCAO group was remarkably smaller than that in the Ast group, but larger than that in the Ast + H89 group (Figure 3C).

Astaxanthin Activated the CAMP/PKA Signaling Pathway to Promote Axonal Regeneration

The cAMP level in the other groups was lower than that in the Sham group at 3 d after operation. Compared with that in the MCAO group,
the cAMP level was lower in the Sham group at 7 and 14 d after the operation, and higher in the Ast group and Ast + H89 group at 7, 14, and 28 d after the operation. Compared with that in the Ast + H89 group, the cAMP level was higher in the Ast group at 14 d after the operation, and there was a statistically significant difference (Figure 4A). The PKA level in the other groups was lower than that in the Sham group at 3 d after the operation. Compared with that in the MCAO group, the PKA level was lower in the Sham group and Ast + H89 group at 7 and 14 d after the operation, and higher in the Ast group at 7, 14, and 28 d after the operation (Figure 4B). The Western blotting results manifested that the PKAc (PKA catalytic subunit) and p-CREB (CREB active molecule) protein expressions in the cortex displayed consistent trends. Compared with those in the MCAO group, the PKAc and p-CREB levels were upregulated in the Ast group at 7, 14, and 28 d after the operation, and downregulated in the Ast + H89 group at 7, 14, and 28 d after the operation (Figures 4C, 4D).

**Discussion**

Ischemic stroke leads to acute and severe neurological dysfunction, which is the leading cause of disability in patients. The nervous system possesses a certain self-repairing capability. Therefore, exploring appropriate intervention measures
Astaxanthin promotes axonal regeneration has been a hot spot in medical research currently. Limb dyskinesia is one of the major clinical manifestations after stroke, and the partial recovery of limb motor function is an evident feature of nerve repair. The Rotarod test and the balance beam footstep error test are commonly-used behavioral tests to evaluate the motor function after brain injury. The results of the two tests revealed that after astaxanthin treatment, the time of footstep errors and the residence time on rotarod of mice with cerebral ischemic infarction were superior to those of mice under spontaneous recovery. The motor function of mice had spontaneous recovery after cerebral infarction, the recovery effect after astaxanthin treatment was better than that under spontaneous recovery, and the recovery of balance and fine motor of mice was earlier than that of coordination and muscle strength.

The neural plasticity after ischemic infarction is a process of building a new structural connection between the tissues around the cerebral lesion and the lesion region. The axonal regeneration is the anatomical basis and the key link

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**Figure 4.** Astaxanthin activated the cAMP/PKA signaling pathway to promote axonal regeneration. A, Analysis of the cAMP level in different groups. B, Analysis of the PKA level in different groups. C, Western Blotting showed protein level of PKAc in different groups. D, Western Blotting showed protein level of p-CREB in different groups. *p<0.05 vs. MCAO group, #p<0.05 vs. Ast group.
of neural remodeling. Studies\textsuperscript{22-24} have found that the surviving pyramidal bundle axons after cerebral infarction of rats can sprout at the distal end of lesion region. Moreover, they found that the sprouting rate of contralateral pyramidal bundle axons is increased, and a large number of axonal fibers can be reorganized along the infarction edge. GAP43 is highly expressed during the axonal growth and differentiation and at the growth cone end of the new axons, and it plays a guiding role in the axonal growth, so it is a reliable marker for axonal growth\textsuperscript{25,26}. In this experiment, the results of immunofluorescence, Western blotting, and RT-PCR showed that the GAP43 expression in the ischemic cortex of mice under spontaneous recovery also began to rise at 7 d after ischemia, indicating that the axonal regeneration has begun after injury. The GAP43 expression in the ischemic cortex of mice treated with astaxanthin was higher than that in mice under spontaneous recovery at 7, 14, and 28 d after ischemia, suggesting that the astaxanthin treatment can stimulate the axonal regeneration in the cortex around infarction, and facilitate the recovery of motor function.

After cerebral infarction, the cortical reorganization occurs around the infarction, and the cortex in the contralateral hemisphere to the injury is also involved in the nerve repair\textsuperscript{27}. BDA is a specific neuronal anterograde tracer, which can be used to observe the growth track of the contralateral new axons on the affected side and axonal density. The cortex of the bilateral cerebral hemispheres in mice injected with BDA was observed via immunohistochemistry. BDA-labeled axonal fibers could be observed in the ischemic cortex, but there were no labeled neurons, indicating that these fibers come from the contralateral cortical neurons near the region around the infarction. Furthermore, the BDA-labeled neurons and axonal fibers were counted via immunofluorescence, and the results showed that the number of labeled neurons and axons in the cortex on the unaffected side had no significant difference among groups except the Sham group, and it was slightly larger in mice treated with astaxanthin. However, there was a significant difference in the number in the ischemic cortex, and the number of labeled axonal fibers in mice treated with astaxanthin was significantly larger than that in other groups, indicating that astaxanthin can stimulate the cortical neurons on the unaffected side to produce more axonal branches extending smoothly to the cortex around the infarction and thereby supporting the neural circuit reconstruction after injury. The colocalization staining of GAP43 and BDA in the cortex around the infarction was basically consistent with the above results, indicating that astaxanthin treatment can promote the reconnection of new axons in the cortex around the infarction.

The cAMP-PKA signaling system has an important influence on regulating neuronal survival and axonal growth\textsuperscript{9,10}. In this experiment, the effect of astaxanthin on promoting neuronal survival and axonal regeneration was inhibited after H89 intervention, indicating that such an effect is related to the PKA signaling pathway. The detection of the cerebral cortex tissues after infarction manifested that the expressions of cAMP and PKA were slightly decreased at 3 d after ischemic infarction compared with those in the Sham group. After astaxanthin treatment, the levels of cAMP and PKA in the cortex of mice with cerebral infarction were increased at 7 d after the operation and reached the peak at 14 d. The results of the Western blotting also demonstrated that the changing trend of PKAc and p-CREB was consistent with that of PKA, indicating that the cAMP/PKA/CREB signaling pathway is activated, and astaxanthin can further stimulate the activation and effect of this pathway, thereby promoting axonal regeneration.

\section*{Conclusions}

We demonstrated that astaxanthin can promote the recovery of motor function in the chronic phase after ischemic infarction and stimulate the axonal regeneration in the cerebral cortex. Its mechanism may be explained as follows: astaxanthin activates the cAMP/PKA/CREB signaling pathway by increasing the cAMP concentration in brain tissues, ultimately promoting the axonal regeneration in the cerebral cortex and improving the motor function.

\section*{Conflict of Interest}

The Authors declare that they have no conflict of interests.

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Abstract: Increasing evidence indicates that environmental tobacco smoke (ETS) impairs cognitive function and induces oxidative stress in the brain. Recently, astaxanthin (ATX), a marine bioactive compound, has been reported to ameliorate cognitive deficits. However, the underlying pathogenesis remains unclear. In this study, ATX administration (40 mg/kg and 80 mg/kg, oral gavage) and cigarette smoking were carried out once a day for 10 weeks to investigate whether the p38 MAPK is involved in cognitive function in response to ATX treatment in the cortex and hippocampus of ETS mice. Results indicated that ATX administration improved spatial learning and memory of ETS mice \( (p < 0.05 \text{ or } p < 0.01) \). Furthermore, exposure to ATX prevented the increases in the protein levels of the p38mitogen-activated protein kinase (p38 MAPK; \( p < 0.05 \text{ or } p < 0.01 \)) and nuclear factor-kappa B (NF-\( \kappa \)B p65; \( p < 0.05 \text{ or } p < 0.01 \)), reversed the decreases in the mRNA and protein levels of synapsin I (SYN) and postsynaptic density protein 95 (PSD-95) (all \( p < 0.05 \text{ or } p < 0.01 \)). Moreover, ATX significantly down-regulated the increased levels of pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor (TNF-\( \alpha \)) (all \( p < 0.05 \text{ or } p < 0.01 \)). Meanwhile, the increased level of malondialdehyde (MDA) and the decreased activities of superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) were suppressed after exposure to ATX (all \( p < 0.05 \text{ or } p < 0.01 \)). Also, the results of the molecular docking study of ATX into the p38 MAPK binding site revealed that its mechanism was possibly similar to that of PH797804, a p38 MAPK inhibitor. Therefore, our results indicated that the ATX might be a critical agent in protecting the brain against neuroinflammation, synaptic plasticity impairment, and oxidative stress in the cortex and hippocampus of ETS mice.

Keywords: astaxanthin; cigarette smoke exposure; p38 MAPK; antioxidant inflammatory; synaptic-associated plasticity

1. Introduction

Environmental tobacco smoke (ETS), the combination of the side-stream smoke emitted from the burning end of a tobacco product and the mainstream smoke exhaled by the smoker, contains more than 6000 chemicals that are harmful to human body and may lead to many serious health problems, such as cognitive impairment and dementia [1,2]. For instance, compared with nonsmokers, smokers are reported to have remarkably decreased prefrontal attention network activity, and such a deficit is...
related with the length of smoking time [3]. Moreover, pregnant women exposed to tobacco smoke may present fetal neurobehavioral damages [4].

Although the pathogenesis of cognitive impairments due to tobacco smoke exposure has not been completely understood, several factors have been implicated such as oxidative stress and inflammation. For instance, long-term exposure to tobacco smoke led to oxidative stress [5]. Oxidative stress, which is mainly attributable to excessive generation of reactive oxygen species (ROS), mediates the activation of the mitogen-activated protein kinases (MAPKs) MAPK signaling cascades, especially the p38 MAPK pathway. As an important member of the MAPK family, p38 MAPK has been demonstrated to play a key role in nuclear factor – kappa B (NF-Kb) activation and pro-inflammatory expression [6]. NF-κB, one of the ubiquitous transcriptional factors, is the main medium that leads to the enlargement of inflammatory responses and then promotes the expression of proinflammatory cytokines like TNF-α and IL-6 [7,8]. Furthermore, researches demonstrate that the tobacco smoking induced oxidative stress and inflammation are involved in brain dysfunction [9]. Meanwhile, excessive ROS and inflammatory cytokines can impair hippocampal structure and function on learning and memory-related synaptic plasticity and neurogenesis [10]. Therefore, we hypothesized that attenuation of oxidative stress and inflammation might reverse the cognitive impairment induced by ETS.

Astaxanthin (ATX), a naturally occurring red carotenoid pigment, is abundant in red yeast Phaffia rhodozyma, green algae Haematococcus pluvialis and many kinds of marine organisms such as salmon and lobsters [11,12]. ATX has hydrophobic polyunsaturated polar structure on both ends of the conjugated olefins structure that facilitate its precise positioning within cell membranes and circulating lipoproteins, before exhibiting potent antioxidant functions as a powerful scavenger of oxygen free radicals so as to decrease oxidative stress and lipid peroxidation [13,14]. Recent studies revealed that ATX can relieve ischemia-related injury in brain tissue by suppressing oxidative stress, glutamate release, and anti-apoptosis [15]. Furthermore, some researches find that ATX can exert neuroprotective effects by weakening neuroinflammation [16]. More excitingly, ATX can attenuate subarachnoid hemorrhage induced neuroinflammation in rats and improve hippocampal plasticity and cognitive functions in male C57BL/6j mice [17]. However, the protective effects of ATX against ETS-induced cognitive decline have not been investigated. Therefore, the current work was designed to evaluate whether ATX can alleviate ETS-induced cognitive decline, and investigate the mechanisms involved.

2. Results

2.1. Effects of ATX Treatment on Exposure to ETS Induced Cognitive Decline

In order to investigate whether ATX could improve the cognitive impairments induced by ETS, we evaluated the learning and memory by the Morris water maze (MWM) test. The trained mice in all groups showed a decrease in mean escape latency during the learning trials (Figure 1A), and from the second day to the fourth day, an apparent elevation appeared in transfer latency in the ETS groups compared with the control group (all \( p < 0.05 \); Figure 1A). On the fifth day, the escape latency significantly elevated in ETS group compared with the control group (\( p < 0.05 \); Figure 1A). Meanwhile, ATX treatment (40 mg/kg and 80 mg/kg) significantly inhibited the elevation of escape latency in the ETS mice (all \( p < 0.05 \); Figure 1A). Administration with ATX (80 mg/kg) alone exhibited no visible difference in the escape latency compared with the control mice (\( p > 0.05 \); Figure 1A). The probe trial was performed on the fifth day. In the ETS mice, the percentage of time spent in the target quadrant (\( p < 0.05 \); Figure 1C) and the number of crossings of the platform area (\( p < 0.05 \); Figure 1D) appeared to decrease in comparison with the control mice, while the decrease of the percentage of time spent in the target quadrant (\( p < 0.05 \); Figure 1C) and the number of crossings of the platform area (\( p < 0.05 \); Figure 1D) were prevented by ATX (40 mg/kg and 80 mg/kg) treatment. ATX (80 mg/kg) alone treated mice presented no visible differences in comparison with the control mice (\( p > 0.05 \); Figure 1C,D), suggesting that ATX itself had no influence on the learning and memory in the control group. The swimming speed exhibited similar performance among the five groups during
the five-days MWM test (all $p > 0.05$, Figure 1B), which indicates that the differences in escape latency, the number of crossings, and the time spent in the target quadrant do not affect the movement defects.

![Figure 1](image_url)

**Figure 1.** Effects of chronic astaxanthin (ATX) treatment on environment tobacco smoke (ETS) induced cognitive decline ($n = 12$). (A) Escape latency appeared during the training and the probe sessions. Data are reported as mean ± SE. (*$p < 0.05$) versus Group Control at the corresponding days; *#$p < 0.05$ versus Group ETS at the corresponding days). (B) The swimming speed among the four groups during the five-day period. Data are reported as mean ± SE ($p > 0.05$). (C) The percentage of time spent in the target quadrant during the probe trial. Data are reported as mean ± SE. (*$p < 0.05$ versus Group Control; *#$p < 0.05$ versus Group ETS). (D) The number of crossings of the platform area. Data are reported as mean ± SE (*$p < 0.05$ versus Group Control; *#$p < 0.05$ versus Group ETS).

2.2. Effects of ATX Treatment on Exposure to ETS Induced Parameters of Oxidative Stress in the Mouse Brain

The MDA levels, SOD activities, CAT activities and GSH levels were detected to investigate whether ATX have the effects on the ETS exposed brain antioxidant system. According to the results described, the ETS mice presented a remarkable increase in the MDA levels ($p < 0.01$, Figure 2A) and a striking decrease in the SOD activities ($p < 0.01$, Figure 2B), CAT activities ($p < 0.01$, Figure 2C) and GSH levels ($p < 0.01$, Figure 2D) in the hippocampus and prefrontal cortex in comparison with the control group. Administration with ATX (40 mg/kg and 80 mg/kg) inhibited the ETS caused elevation of MDA levels ($p < 0.05$ or $p < 0.01$; Figure 2A) and prevented the ETS caused decrease of SOD activities ($p < 0.05$ or $p < 0.01$; Figure 2B), CAT activities ($p < 0.05$ or $p < 0.01$; Figure 2C) and GSH levels ($p < 0.05$ or $p < 0.01$; Figure 2D) in the hippocampus and prefrontal cortex. ATX (80 mg/kg) alone treatment presented no difference in these parameters of oxidative stress in comparison with the control mice in the hippocampus and prefrontal cortex ($p > 0.05$; Figure 2). These results indicated that chronic exposure to ETS caused oxidative stress in mice, and ATX treatment could attenuate the ETS caused oxidative stress.

2.3. Effects of ATX Treatment on Exposure to ETS-Induced Inflammation in the Hippocampus and Prefrontal Cortex

Inflammatory response is closely linked to the pathogenesis of cognitive disorder, which damages hippocampal synaptic plasticity by increasing the levels of pro-inflammatory cytokines. Thus, the effects of ATX on ETS induced alteration of the levels of inflammatory factors (such as TNF-α and IL-6) in the brain were tested by ELISA. The levels of TNF-α ($p < 0.01$; Figure 3A) as well as IL-6 ($p < 0.01$; Figure 3B) were found to be increased remarkably in the hippocampus and cortex in ETS mice in comparison with control mice, while ATX (40 mg/kg and 80 mg/kg) administration attenuated...
the ETS induced increase in the levels of TNF-α (p < 0.05 or p < 0.01; Figure 3A) and IL-6 (p < 0.05 or p < 0.01; Figure 3B) in the hippocampus and cortex. ATX (80 mg/kg) treatment alone did not change inflammation levels in the hippocampus and cortex in comparison with the control group (p > 0.05; Figure 3A and B). These results inferred that ETS caused inflammatory response, and ATX could inhibit ETS caused inflammatory response.

2.4. Effects of ATX on the Expressions of NF-κB p65 in the Hippocampus and Prefrontal Cortex

NF-κB p65 expression was carried out to study the potential mechanisms of the neuroprotective changes in ATX treatment of ETS caused cognitive impairment. As shown in Figure 4, there was an obvious enhancement in NF-κB p65 levels (p < 0.01) in the hippocampus and cerebral cortex in ETS mice compared with control mice and this enhancement was repressed by ATX (40 mg/kg and 80 mg/kg) treatment (p < 0.05 or p < 0.01). Treatment with ATX (80 mg/kg) alone exhibited no difference compared to the control mice (p > 0.05). These results suggested that ETS enhanced the
levels of the NF-kB, and ATX administration could prevent the enhancement of the levels of the NF-κB p65.

Figure 4. Effects of ATX on the expressions of NF-κB p65 in the cortex and hippocampus (n = 12). The levels of NF-κB p65 in the hippocampus and cortex of mice. Data are reported as mean ± SE. (** p < 0.01 versus Group Control; * p < 0.05 versus Group ETS; ## p < 0.01 versus Group ETS).

2.5. Effects of ATX on the Expressions of p38 MAPK and p- p38MAPK in the Hippocampus and Prefrontal Cortex of ETS Mice

The protein expression of total-p38 MAPK and p- p38 MAPK in the hippocampus and prefrontal cortex were tested by Western blot and the results are shown in Figure 5. In the ETS mice, the levels of phosphorylated p38 MAPK were remarkably increased in the hippocampus and cerebral cortex in comparison with the control mice (p < 0.01), while the increased levels of phosphorylated p38 MAPK were prevented in the ATX (40 mg/kg and 80 mg/kg) mice (p < 0.05 or p < 0.01). ATX (80 mg/kg) alone groups exhibited no obvious difference compared to the control group (p > 0.05). The levels of the total-p38 MAPK exerted no obvious differences among all groups in the hippocampus and cerebral cortex (p > 0.05). These results indicated that ETS caused the excessive activation of p38 MAPK, and ATX could inhibit the ETS caused the activation of p38 MAPK.

2.6. Effects of ATX on the Expression of SYN mRNA and PSD-95 mRNA in the Mouse Brain of ETS mice

SYN and PSD-95 are two major synaptic associated proteins that can directly or indirectly affect cognitive function [18]. Accordingly, reverse transcriptase-PCR (RT-PCR) was used to estimate the levels of SYN and PSD-95 mRNA. In the ETS mice, the expression of SYN mRNA (p < 0.01; Figure 6A) and PSD-95 mRNA (p < 0.01; Figure 6B) were markedly down-regulated in the hippocampus and cortex compared with the control mice, while this down-regulation of the SYN mRNA (p < 0.05 or p < 0.01; Figure 6A) and PSD-95 mRNA (p < 0.05 or p < 0.01; Figure 6B) levels were elevated by ATX.
(40 mg/kg and 80 mg/kg) administration. ATX (80 mg/kg) alone had no influence on the expression of SYN mRNA ($p > 0.05$; Figure 6A) and PSD-95 mRNA ($p > 0.05$; Figure 6B) in comparison with the control mice. These results inferred that ETS led to a reduction of the SYN mRNA and PSD-95 mRNA, and ATX could reverse this change.

![Figure 5](image)

**Figure 5.** Effects of ATX on the expressions of p38 and p-p38 in the cortex and hippocampus ($n = 12$). The levels of p-p38 in the hippocampus and cortex of mice. Data are reported as mean ± SE. (** $p < 0.01$ versus Group Control; # $p < 0.05$ versus Group ETS; ## $p < 0.01$ versus Group ETS).

### 2.7. Effects of ATX on the Expression of Synaptic Proteins in the Mouse Brain

In order to detect whether ATX could protect synaptic plasticity from ETS impairment Western blot was used to examine the expression of SYN and PSD-95 proteins in the hippocampus and cortex. In the ETS mice, SYN ($p < 0.01$; Figure 7A) and PSD-95 ($p < 0.01$; Figure 7B) were noticeably reduced in the hippocampus and cortex compared with the control mice, while treatment with ATX (40 mg/kg and 80 mg/kg) inhibited this reduction of both SYP ($p < 0.05$ or $p < 0.01$) and PSD-95 ($p < 0.05$ or $p < 0.01$) expressions in ETS mice. Administration with ATX (80 mg/kg) alone presented no difference in SYP and PSD-95 expressions compared with the control mice ($p > 0.05$). These results inferred that ATX could prevent ETS induced changes of the SYN and PSD-95 protein expressions.
Figure 6. Effects of ATX on the expression of SYN mRNA and PSD-95 mRNA in the mouse brain (n = 12). (A) The levels of SYN mRNA. Data are reported as mean ± SE. (** p < 0.01 versus Group Control; * p < 0.05 versus Group ETS; # p < 0.01 versus Group ETS). (B) The levels of PSD-95 mRNA. Data are reported as mean ± SE. (** p < 0.01 versus Group Control; * p < 0.05 versus Group ETS; # p < 0.01 versus Group ETS).

Figure 7. Effects of ATX on the expression of synaptic proteins in the mouse brain (n = 12). (A) The levels of SYN in the hippocampus and cortex of mice. Data are reported as mean ± SE. (** p < 0.01 versus Group Control; * p < 0.05 versus Group ETS; # p < 0.01 versus Group ETS). (B) The levels of PSD-95 in the hippocampus and cortex of mice. Data are reported as mean ± SE. (** p < 0.01 versus Group Control; * p < 0.05 versus Group ETS; # p < 0.01 versus Group ETS).

2.8. Effects of ATX on the Structure and Morphology of the Hippocampal Neurons

Microphotographies of the cerebral cortex and the hippocampal CA1 subfield in each group are shown in Figure 8. In the ETS group, no obvious differences were observed in the neurons in the cerebral cortex in comparison with the control group. Meanwhile, no obvious differences were found in intact neuron counts in the cerebral cortex among the groups (Figure 8B). In contrast, in the hippocampal CA1 subfield of ETS mice, the neurons appeared in a noticeably wrinkled, irregular pattern, and a weak staining effect, and most Nissl bodies were lost, which inferred that extensively they were injured or dead (Figure 8A). Also, a significant decrease in the number of surviving neurons was observed in the ETS mice as compared to the control mice, while ATX (40 mg/kg and 80 mg/kg) treatment remarkably attenuated this decrease in ETS mice. Additionally, the ATX (80 mg/kg) group
alone and the control group showed no difference in the number of surviving neurons, which revealed that ATX itself had no effect on the neurons in different areas of brain.

**Figure 8.** Effects of ATX on the structure and morphology of the hippocampal neurons \((n = 12)\). Nissl stained neurons in the hippocampal CA1 subfield (A) and cortex (B). Bar = 20 \(\mu\)m.

2.9. Molecular Docking Studies

The interactions between ATX and human p38 and the interaction between human p38 alpha and p38 inhibitor PH797804 are shown as Figure 9. The p38 alpha and ATX docking pocket was formed by the residues of Glu-71, Leu-167, Phe-169, Leu-171, Thr-175, Arg-49, Leu-108, Met-109, Thr-106 and Leu-104, and a hydrogen bond was formed to Glu-71 from helix C. Meanwhile, another two hydrogen bonds were formed between the side chain of Arg-49 and the opposite end of ATX. p38 alpha and
p38 inhibitor PH797804 docking pocking were formed by a hydrogen bond of Gly-110. The docking result demonstrated that ATX occupied the active site of the p38 and generated an interaction with surrounding amino acids like p38 inhibitor PH797804.

**Figure 9.** Molecular docking model for ATX (green and stick) with the active site of the ATP pocket of p38 alpha and the p38 alpha with the p38 inhibitor PH797804. **(A)** The whole picture of the molecular docking model for ATX with the p38 alpha. **(B)** Molecular docking model for ATX with the p38 alpha. Highlighting the hydrogen bonds (red dashed lines) coordination between the oxygen atoms in ATX and residues Glu-71 and Arg-49. **(C)** Molecular docking model for p38 MAPK with p38 MAPK inhibitor PH797804 binding pocket. The red color bonds indicate hydrogen bonds.
3. Discussion

According to the present research, chronic ATX administration reversed the ETS-induced cognitive deficits of mice. Notably, we found that ATX administration normalized the oxidative stress markers, decreased the levels of inflammatory cytokines, phospho-p38 MAPK, and NF-κB p65 proteins in the hippocampus and prefrontal cortex. In addition, the levels of SYN and PSD-95 were increased in the prefrontal cortex and hippocampus of ATX-treated mice. What is more important is that p38 MAPK may be the key factor in the reduction of cognitive deficits.

Previous studies have reported that the capacity for learning and memory were impaired by cigarette smoke exposure [19,20]. However, the mechanism of ETS-induced cognitive impairment remains unclear. ROS are closely related with neuroinflammation and synaptic plasticity impairment [21]. Chronic cigarette smoke exposure induced an excessive ROS generation followed by the loss of the dynamic balance between ROS generation and elimination [16,22]. As a marine bioactive compound, ATX is reported to have antioxidant and anti-inflammation properties [16]. The present data indicated that ETS induced impairment in learning and memory function was improved by ATX treatment. To our knowledge, it is the first report that ETS-induced cognitive deficits can be improved by ATX treatment and the p38 MAPK may be the key factor in the reduction of cognitive deficits.

Generally, the MWM test is widely applied to measure the spatial learning of rodents [23]. Some researches demonstrate that the performance in the MWM test is usually related with both neurotransmitter systems and drug effects [24]. Several studies have confirmed that long term exposure to tobacco smoke could cause cognitive deficits [19,25]. Importantly, ATX can enhance cognitive function and attenuate depression-like behavior. So, we used MWM to observe the ETS-induced cognitive deficits and explore the therapeutic effect of ATX. The result of MWM indicated that the mice exposed to ETS showed enhanced escape latency and reduced time spent in the target quadrant (revealing an impairment of spatial learning and memory), which is consistent with published results [26]. Above all, long-term administration with low or high doses of ATX markedly reversed these behavioral changes, suggesting that ATX is the potential to protect ETS-induced cognition damage.

What is well recognized is that oxidative damage plays a crucial role in many brain dysfunction diseases [27]. Importantly, the brain is particularly vulnerable to oxidative stress because of a relatively high production rate of ROS without commensurate levels of antioxidative defense [28]. Tobacco smoke contains a large number of ROS which can permeate the blood brain barrier and mobilize the antioxidant defenses [29]. In the current research, we found an elevation of MDA, and a reduction of GSH, SOD, and CAT activities in the cerebral cortex and hippocampus of ETS mice, which is consistent with published results [30]. It has been proved that several flavonoids have strong antioxidant properties and improve memory and learning [31]. Moreover, treatment with ATX could decrease the MDA level and increase the SOD level in aging rats [32]. Our results showed that, the MDA level was suppressed, but GSH content, SOD, and CAT activity were raised when chronic administration with ATX in the hippocampus and prefrontal cortex of ETS mice. Consequently, these results support the hypothesis that ATX can inhibit the chronic ETS-induced pro-oxidant–antioxidant disequilibrium contributing to cognition improvement.

p38 MAPK as a stress-activated kinase, is sensitive to various exogenous and endogenous stimulations, and highly responded to oxidative stress and proinflammatory cytokines [33]. In addition, recent studies have found that the activation of the p38 MAPK signaling pathway is closely related with neuronal death or apoptosis, which may be the main reason of cognitive dysfunction [34]. In the current work, we found that the phosphorylation level of p38 MAPK was remarkably raised in the hippocampus and prefrontal cortex of ETS mice. And the chronic ATX administration attenuated the p38 MAPK phosphorylation level. Thus, we speculate that the cognition impairment of ETS mice may contribute to oxidative stress and the activation of p38 MAPK, where the activation of p38 MAPK may be more important.
It is well established that inflammation and oxidative stress are intricately interrelated. Oxidative stress is considered to be a crucial factor in regulating proinflammatory signaling pathways [35]. Long-term exposure to ETS induced oxidative stress and the activation of NF-κB followed by the release of the pro-inflammatory [36]. In addition, many studies confirm that the activation of NF-κB and the release of inflammation cytokines play a key part in the cognitive dysfunction that may explain cognitive decline [37]. In the current research, we also detected that the mice exposed to tobacco smoke showed up-regulated levels of NF-κB p65 and TNF-α and IL-6. However, chronic treatment with ATX remarkably suppresses the expression of NF-κB p65 and attenuates the excessive release of TNF-α and IL-6 [38].

The alterations of structural plasticity of dendrites and spines in the hippocampus and prefrontal cortex were found as a result of cognition deficits [39]. Research indicates that morphological alterations in the brain development of mice exposed to smoke may disrupt neural prediction [40]. Generally, synaptic plasticity is associated with the synapse related proteins, including presynaptic SYN and postsynaptic PSD-95 [41]. In our research, the reduction in the expression of synaptic proteins was observed in ETS-exposed mice, which may result in cognitive impairment. However, the reduction in both SYN and PSD-95 levels in ETS exposure mice was remarkably overturned, by chronic administration with ATX. Both SYN and PSD-95 were regulated by the inflammatory response caused by p38 MAPK and NF-κB p65. Therefore, these neurochemical findings imply that the neuroprotective response of ATX is attributable to reducing the phosphorylation level of p38 MAPK and relieving inflammatory responses. Thus, cognitive impairment in ETS-exposed mice can be improved by increasing the level of plastic-related proteins (SYN and PSD-95).

In conclusion, these findings manifest that ATX exerted protective effects on the cognition decline caused by ETS in mice. These improvements in the behaviors and neurochemicals implied that supplementation with ATX-enriched food may be an effective novel therapy and provide a hopeful mitigation to chronic ETS-induced cognition decline. Administration of ATX reduced oxidative stress and inflammatory responses, as well as enhanced the synapse-related proteins in the hippocampus and prefrontal cortex of ETS mice, and p38 MAPK plays an important role in the protection process. Therefore, our results provide ideals for further studies on the anti-inflammatory or antioxidant aspects of ATX and ATX derivatives in CNS related diseases in the future.

4. Materials and Methods

4.1. Reagents

ATX (97% purity) was purchased from Xi’an Fengzu Biotechnology Co., Ltd (Shaanxi, China) and dissolved in olive oil (1 mL/kg) immediately before use. MDA, SOD, GSH, CAT, and BCA assay kits were obtained from Nanjing Jiancheng Biotechnology Co., Ltd (Nanjing, China). Antibodies against phospho-p38 MAPK (T180/Y182), p38 MAPK, SYN, PSD-95 and NF-κB p65 were from Cell Signaling Technology Inc., (Danvers, MA USA) and β-actin was from ZSGB-BIO, Beijing, China. All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

4.2. Animals

Adult male Kunming mice weighing between 18 and 22 g were purchased from the Laboratory Animal Center, Xuzhou Medical University. The whole experimental schedule was depicted in Figure 10. The mice were housed with a 12 h light/dark cycle and free access to food and water under controlled temperatures 22 ± 2 °C and humidity 50 ± 10%. The animals were sacrificed within 24 h after the final test. All animal experiments in the current study were conducted in accordance with the Animal Ethics Committee, Xuzhou Medical University, China, and followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (Ethical approval number: XZMC2014-AN-39).
The mice were randomly divided into five groups according to their corresponding treatments \((n = 12)\): (1) an ETS group: mice were exposed to ETS once a day for 2 h with an interval of 10 min between each cigarette, using 8 cigarettes per day for 10 consecutive weeks; (2) an ETS+ATX-L group: mice were exposed to ETS once a day for 2 h with an interval of 10 min between each cigarette, using 8 cigarettes followed by treatment with a low dose of ATX \((40 \text{ mg/kg})\) once a day for 10 consecutive weeks; (3) an ETS+ATX-H group: mice were exposed to ETS once a day for 2 h with an interval of 10 min between each cigarette, using 8 cigarettes followed by treatment with a high dose of ATX \((80 \text{ mg/kg})\) per day for 10 consecutive weeks; (4) an ATX group: mice were treated with 80 mg/kg ATX alone per day for 10 consecutive weeks; (5) a control group: under normal conditions with an equal volume of olive oil as ATX treatment once a day for 10 consecutive weeks. ATX was dissolved in olive oil before administration. Either ATX or the equal volume of olive oil was administered by oral gavage.

4.3. Smoke Generation

In the current study, smoke was generated according to previous descriptions \([42]\). Each cigarette contains 10 mg tar, 0.8 mg nicotine, and 10 mg carbon monoxide. After the mice were placed within a chamber \((56.4 \text{ cm} \times 38.5 \text{ cm} \times 37.1 \text{ cm})\), four cigarettes (Jiangsu Tobacco Industrial Co. Ltd., China) were lit at one time and the chamber was shut down immediately, leaving a small hole \((371 \text{ mm} \times 40 \text{ mm})\) in both ends for ventilation, and the cigarettes were burned up within 15 min. In order to keep an air flow inside the chamber, the smoke generated within the chamber was pumped by a noiseless extractor fan. The diluted side-stream smoke exposed to mice was adopted to imitate the ETS experienced for non-smokers.

4.4. Morris Water Maze (MWM)

The Morris water maze test was performed according to previous descriptions \([43,44]\). Mice were trained in a black circular pool \((120 \text{ cm in diameter and } 60 \text{ cm in height})\) filled with water \((20–22 ^\circ \text{C})\). The pool was divided into four quadrants with a clear 10 cm diameter escape platform hidden 1.5 cm beneath the surface in one of the quadrants. Training trials were conducted in the first consecutive four days, and the escape latency was recorded according to the time spent to reach the hidden platform. Then a probe trial was performed on the fifth day and the hidden platform was removed. The total time spent in each target quadrant was recorded.

4.5. Measurement of Oxidative Stress

After the behavioral assessments, the mice were sacrificed. The hippocampus and prefrontal cortex were dissected and homogenized \((1:9 \text{ w/v})\) with cold normal saline \((4 ^\circ \text{C})\) to prepare 10%
cerebral homogenate in an ice bath. The homogenized tissue was centrifuged at 4000 rpm at 4 °C for 10 min and the supernatant was collected for the following tests.

4.5.1. Determination of Lipid Peroxidation

The MDA level was measured by supernatants reacted with thiobarbituric (TBA) to form thiobarbituric acid reactive substances using a commercial kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [45] and the absorption was determined at the wavelength of 532 nm.

4.5.2. Determination of SOD Activity

The activity of SOD was assayed according to the method previously described [46]. Xanthine reacts with xanthine oxidase to produce superoxide radicals which then react with nitro-blue tetrazolium (NTB) to form a colored formazan dye. The amount of formazan generated was determined by the absorption at the wavelength of 550 nm. One unit of enzyme was defined as the amount of enzyme required at an inhibition rate of 50%. Enzyme specific activity was expressed in units per milligram protein.

4.5.3. Determination of CAT Activity

The activity of CAT was assayed based on the method previously described [47]. Briefly, 0.1 mL of supernatant of tissues in hippocampus and cortex was added to 1.91 mL of 50 mmol/L phosphate buffer (pH 7.0). Then 1 mL freshly prepared 30 mmol/L H$_2$O$_2$ was added to start the reaction. The decrease in H$_2$O$_2$ content was determined by the absorption at the wavelength of 240 nm.

4.5.4. Determination of GSH

The concentration of GSH was assayed according to a previous method [48]. In brief, 160 μL of supernatant of tissues in hippocampus and cortex was added to 2 mL of Ellman’s reagent (5,5'-dithiobis [2-nitrobenzoic acid] 10 mM, NaHCO$_3$ 15 mM). The mixture was incubated at room temperature for 5 min and the absorption was measured at the wavelength of 412 nm.

4.6. Enzyme-linked Immunosorbent Assay (ELISA)

The frozen brain cortex and hippocampal tissues were homogenized in ice-cold normal saline and centrifuged at 12,000 rpm at 4 °C for 5 min. The supernatants were then collected, and the total protein concentration was assayed using Micro BCA procedures (Beyotime Institute of Biotechnology, Shanghai, China). On the basis of the manufacturer’s instructions, enzyme-linked immunosorbent assay (ELISA) kits (Immuno-Biological Laboratories Co., Ltd., Japan) were used to quantify TNF-α and IL-6 in the supernatants.

4.7. Western Blotting

The frozen cerebral cortex and hippocampus tissues were homogenized in ice-cold extraction buffer (20 mM Tris-HCl buffer, pH 7.6, 150 mM NaCl, 2 mM EDTA-2Na, 50 mM sodium fluoride, 1 mM sodium vanadate, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 1 mg/mL aprotinin, and 1 mg/mL leupeptin). The resultant homogenates were centrifuged at 10000× g for 10 min at 4 °C to obtain the final supernatants. Nuclear and cytoplasmic extracts for Western blot analysis were extracted using a nuclear/cytoplasmic isolation kit (Beyotime Institute of Biotechnology, Shanghai, China). Pierce BCA Protein Assay Kit (ibid.) was used to determine protein concentrations. Equal amounts of protein (20 μg) for each sample were separated by SDS–PAGE and transferred onto nitrocellulose membranes. And 5% skim milk powder in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) was used to block the membranes at 25 °C for 2 h, before incubation with the primary antibodies to NF-κB p65 (1:1000), p38 MAPK (1:1000), phospho- p38 MAPK (1:1000), SYN (1:1000), and PSD-95 (1:2000) and β-actin (1:1000) at 4 °C overnight. Then, the membranes were washed three times every 15 min with
TBST and then incubated with the secondary horseradish peroxidase-linked anti-rabbit (1:1000) or anti-mouse (1:1000) antibodies (ZSGB-BIO, Beijing, China) at 37 °C for 1 hour. Bands were scanned, and the density was analyzed by the Quantity One analysis software (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were performed based on our former researches [49].

4.8. Reverse Transcriptase-PCR (RT-PCR)

The assay was performed based on previous researches [44,48]. The total RNA was extracted using trizol reagent. A High Capacity RNA-to-cDNA kit was applied to synthesize cDNA. The sequences of the forward and reverse primers for SYN, PSD-95 and the housekeeping gene β-actin (Sangon Biotech Co. Ltd., Shanghai, China) are shown in Table 1. Electrophoresis on a 1% agarose gel was used to separate amplified products followed by photography for visualization under a UA trans-illuminator. In order to verify reproducibility, duplicate reaction was performed. The values obtained for the target gene expression were normalized to β-actin and quantified relative to the expression in the control samples. The products were analyzed with densitometry using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA, USA).

| Target mRNA Sequences | Primer Sequence | Annealing Tm (°C) |
|-----------------------|-----------------|-------------------|
| β-actin               | 5'-ATGGTCACGCAAGTCC-3' | 59 |
|                       | 5'-GAGACCTTCAAACCCCAGC-3' | |
| SYN                   | 5'-TCTTCGCAAGAAAGTACC-3' | 200 |
|                       | 5'-CCTTGCAATGTGTCCTGTTGTC-3' | |
| PSD-95                | 5'-CCTGGACATCACAACTCAT-3' | 324 |
|                       | 5'-ACACATTGAACACGAGAT-3' | |

4.9. Histological Analysis

After the behavioral test, mice were immediately anesthetized with sodium pentobarbital (50 mg/g, i.p. injection) and then perfused with ice-cold normal saline followed by 4% paraformaldehyde via the left ventricle. The whole brain was removed and fixed in 4% paraformaldehyde, then in 15% cane sugar for 24 h, followed by dehydration in 30% cane sugar for 12 h. For histological analysis with Nissl’s staining, all specimens were frozen and cut into consecutive coronal sections (30 μm in thickness). The number of intact cells in the cerebral cortex and hippocampal CA1 subfield were counted by an investigator blinded to sample identity, and the average value from adjacent two sections was used for each animal. Data were represented as cells per mm². The histological analysis was performed as previous research described [50].

4.10. Molecular Docking Studies

In order to investigate the possible binding modes of ATX with human p38 alpha and human p38 alpha with p38 inhibitor PH797804, a molecular docking study was carried out using the Sybyl v7.1 program package (Tripos International, St. Louis, MO). The three-dimensional structure of human p38 and p38 inhibitor PH797804 alpha were taken from the Protein Data Bank (PDB ID: 4l8m; http://www.rcsb.org/), hydrogen atoms were added to the crystallographic structures and all the water were removed subsequently. The energy of human p38 and p38 inhibitor PH797804 alpha were minimized, before ATX had been docked into the active site of the ATP pocket of p38 alpha.

4.11. Statistical Analysis

All values are expressed as the mean ± SEM and analyzed by SPSS v16.0 (SPSS, Inc., Chicago, IL, USA). Differences between the groups were assessed by the one-way ANOVA and the Turkey’s test. Significant differences were represented as * p < 0.05.
Author Contributions: X.Y. and Y.L. designed the experiments. X.Y. and A.-L.G. wrote the manuscript. X.Y., A.-L.G., Y.-P.P., X.-J.C., T.X., X.-R.L., J.L. and Y.-Y.Z. performed experiments and analyzed data.

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High-Dose Astaxanthin Supplementation Suppresses Antioxidant Enzyme Activity during Moderate-Intensity Swimming Training in Mice

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Abstract: Exercise-induced reactive oxygen and nitrogen species are increasingly considered as beneficial health promotion. Astaxanthin (ASX) has been recognized as a potent antioxidant suitable for human ingestion. We investigated whether ASX administration suppressed antioxidant enzyme activity in moderate-intensity exercise. Seven-week-old male C57BL/6 mice (n = 8/group) were treated with ASX (5, 15, and 30 mg/kg BW) combined with 45 min/day moderate-intensity swimming training for four weeks. Results showed that the mice administrated with 15 and 30 mg/kg of ASX decreased glutathione peroxidase, catalase, malondialdehyde, and creatine kinase levels in plasma or muscle, compared with the swimming control group. Beyond that, these two (15 and 30 mg/kg BW) dosages of ASX downregulated gastrocnemius muscle erythroid 2p45 (NF-E2)-related factor 2 (Nrf2). Meanwhile, mRNA of Nrf2 and Nrf2-dependent enzymes in mice heart were also downregulated in the ASX-treated groups. However, the mice treated with 15 or 30 mg/kg ASX had increased constitutive nitric oxidase synthase and superoxide dismutase activity, compared with the swimming and sedentary control groups. Our findings indicate that high-dose administration of astaxanthin can blunt antioxidant enzyme activity and downregulate transcription of Nrf2 and Nrf2-dependent enzymes along with attenuating plasma and muscle MDA.

Keywords: astaxanthin; antioxidant; oxidative stress; chronic exercise; physical adaption

1. Introduction

For decades, regular, nonexhaustive physical exercise has been considered beneficial for improving health and physical fitness. Many studies show that chronic physical exercise can prevent several chronic diseases (e.g., cardiovascular disease, diabetes, cancer, hypertension, obesity, depression, and osteoporosis) and premature death. However, during high-intensity exercise, reactive oxygen and nitrogen species (RONS) are yielded simultaneously, which may damage important macromolecules such as lipids, protein, and DNA [1,2]. That said, organisms have evolved complicated endogenous antioxidant defense mechanisms to minimize the potential damage caused by increased oxidative stress [3]. Key antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT), synergistically eliminate damaging free radical species. Two nuclear...
factors, erythroid 2p45 (NF-E2)-related factor 2 (Nrf2) and kelch-like ECH-associated protein 1 (Keap1), regulate the transcription of phase II detoxifying enzymes in accordance with oxidative stress, thereby maintaining cellular homeostasis in vivo [4,5]. Many Nrf2-dependent antioxidant enzymes, which have antioxidant-response elements (AREs) in untranslated gene regions, are activated by Nrf2. These enzymes embrace glutamate-cysteine ligase catalytic subunit (GCLC) and glutamate-cysteine ligase modifier subunit (GCLM), which are the first-rate limiting enzymes of glutathione synthesis [6,7]; NAD(P)H quinone dehydrogenase 1 (NQO-1), which prevents the one-electron reduction of quinones [8]; Heme oxygenase 1 (HMOX-1), which cleaves heme to form biliverdin [9]. Although an overabundance of RONS, generated within cells, can be counterproductive and overwhelm the endogenous antioxidant defense system, an optimal amount of RONS produced during exercise can be beneficial for exercise adaption and overall health [10–12]. Over the years, many studies have shown that oral administration of vitamin C or vitamin C and E complex decreases muscle mitochondrial biogenesis and hampers physical adaptations in endurance performance [13–17]. However, other studies have shown that antioxidant treatment does not eliminate the beneficial effects of exercise [18–22]. Therefore, the benefits of antioxidant supplementation in exercise training have become a controversial topic.

Astaxanthin (ASX) is a xanthophyll carotenoid, which is a fat-soluble red pigment found in several species, such as microalgae, crustacea, fish, and birds [23]. According to the previous studies, ASX can directly scavenge peroxyl (ROO•), alkoxyl (RO•), and singlet oxygen in a dose-dependent way, but does not directly affect transcription of antioxidant enzymes [24]. Many other studies have documented that dietary consumption of ASX can prevent or reduce the risk of various medical conditions in humans and animals [25]. In addition, long-term supplementation of ASX in mice also delays time to exhaustion during exercise [26–30]. Hence, ASX is considered a potent antioxidant utilized as a nutritional supplement for physical exercise participants [31]. Nevertheless, previous studies have revealed that antioxidant supplementation can blunt the expression of antioxidant enzymes; this may decrease muscle adaption capability following exercise [16,17]. As such, it is our contention and hypothesis that ingestion of high doses of the antioxidant ASX may blunt antioxidant enzymes in vivo during bouts of regular physical exercise. Therefore, the purpose of this study was to investigate the relationship between antioxidant status and the dosage of ASX supplementation using a moderate-intensity exercise mouse swimming model.

2. Materials and Methods

2.1. Astaxanthin Source, Animals, and Experimental Design

Astaxanthin samples (Cat. No. SML 0982) were purchased from Sigma-Aldrich Co. LLC (Saint Louis, MO, USA) and dissolved in olive oil, which was prepared in advance and stored at –20 °C. Forty male C57BL/6 mice (7 weeks old) with weights ranging from 20~25 g were obtained from the Experimental Animal Centre of Zhejiang Province, China, and four mice in each cage were housed in the Animal Centre of Ningbo University College of Medicine (Ningbo, China). All mice were acclimatized for one week in an air-conditioned (22 ± 2 °C and approximately 60% RH) room under a 12 h light/dark cycle (lights on from 07:30 to 19:30 h) with food and water provided ad libitum. The composition of mouse feed is listed in a Supplementary Materials.

The mice were randomly divided into five groups, consisting of a sedentary control (SEC) group, a swimming control (SWC) group, and three swimming plus ASX (SA) groups; each group was assigned eight mice. For eliminating the background effect of olive oil acting on mice, the mice in the SEC and SWC groups were orally administered 0.1 mL of olive oil by gavage each day. The mice in the three SA groups were orally treated with 0.1 mL of the mixture of ASX and olive oil by gavage and participated in a supplementation regime with dosages of ASX 5 mg/kg Body Weight (BW; SA5), 15 mg/kg BW (SA15), and 30 mg/kg BW (SA30), respectively. The supplemented quantity of ASX was based on published work [26,28]. However, some modifications were made for evaluating ASX concentrations
in larger quantities during this experiment. All the mice were fed with an ASX mixture or olive oil two hours prior to the initiation of swimming training.

The mice swimming model was based on previous research with slight modifications [32,33]. The mice in the swimming groups performed chronic swimming training in a heated water tank (31 ± 2 °C), five times a week for four weeks. No weight loads were used. During the four-week training period, the durations of the swimming tasks were gradually increased from an initial 10 min/time to a final level of 45 min/time, with an increasing duration of 5 min. After four weeks of training, all experimental mice were sacrificed at 24 h postexercise (Figure 1). The experimental protocol used was in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Ethical Committee of Animal Use and Protection at Ningbo University Health Science Centre. The handling of animals was also in accordance with the consensus author guidelines on animal ethics.

2.2. Sample Collection and Preparation

Blood samples were immediately collected from an eye socket vein and preserved in EDTA-tubes. The blood samples were centrifuged at 3000×g for 5 min, and the plasma aliquots were subsequently stored at −80 °C for biochemical analysis (enzyme activity and lipid peroxidation). The gastrocnemius muscles of the mice were isolated immediately, and each one was divided into two portions. One portion, used for total RNA extraction, was immersed in RNAlater reagent (Cat. No. AM7020, Ambion, city, country) and stored at −80 °C for future analysis. The other portion was wrapped in aluminum foil and stored at −80 °C. The gastrocnemius muscle tissue used for the determination of enzyme activity or lipid peroxidation was homogenized in saline in an ice bath. The homogenated samples were centrifuged at 3500×g for 10 min and the supernatants were placed into new EP tubes and immediately stored at −80 °C for future analysis.

2.3. Antioxidant Enzymes and Malondialdehyde Assays

Total superoxide dismutase (SOD) activity in plasma was determined using a superoxide dismutase (SOD) assay kit (Cat. No. A001-3, Nanjing Jiancheng Biotech Company, Nanjing, China). The glutathione peroxidase (GPx) activity both in plasma and muscle was assayed using a glutathione peroxidase assay kit (Cat. No. A005, Nanjing Jiancheng Biotech Company). Catalase (CAT) activity in muscle was measured using a catalase assay kit (Cat. No. A007-1, Nanjing Jiancheng Biotech Company). Malondialdehyde (MDA) concentrations in plasma or muscle tissue were obtained using a

Figure 1. The experimental design and flow diagram for evaluation of astaxanthin.
Microscale malondialdehyde assay kit (Cat. No. A003-2, Nanjing Jiancheng Biotech Company). All assays were performed and completed following the manufacturer’s instructions.

2.4. Nitric Oxide Synthase and Creatine Kinase Assays

Plasma nitric oxide synthase activity was assayed using the Nitric Oxide Synthase typed assay kit (Cat. No. A014, Nanjing Jiancheng Biotech Company). The kit was used to quantify total nitric oxide synthase (tNOS), inducible nitric oxide synthase (iNOS), and constitutive nitric oxide synthase (cNOS) activity. cNOS activity was calculated by the value of tNOS subtracted by that of iNOS. Plasma creatine kinase (CK) activity was measured by a creatine kinase assay kit (Cat. No. A032, Nanjing Jiancheng Biotech Company). All assays were conducted in accordance with the manufacturer’s instructions.

2.5. Analysis of Nrf2 and Nrf2-Dependent Gene Transcription in Gastrocnemius and Heart

Total RNA was extracted from the gastrocnemius muscle or heart from different groups using TransZol Up Plus RNA Kit (Cat.No. ER501, Tansgen Biotech, Beijing, China), following the manufacturer’s instructions. Two micrograms of each total RNA sample aliquot was treated with RNase free DNase I (Takara, Dalian, China) and desalted before the first strand cDNA synthesis using RNeasy MinElute Cleanup Kit (Cat.No. 74204, QIAGEN, Germany). The first-strand cDNA was synthesized using HiFiScript gDNA Removal cDNA Synthesis kit (Cat.No. CW2582M, CWBIO, China). The quantitative PCR was performed using TB Green Premix Ex Taq II kit (Takara, Japan). To evaluate PCR efficiency, ten-fold serial dilutions of target gene plasmid cDNA, were used to create standard curves for each gene. The specific primers (5’ to 3’) were GCTCTATGCTGAACTCCAA and TTTGGCTAAGCTATCTCGT for Nrf2; CCATGTTCACCAACGGCCTTC and CGTGACAGAACACTTTCG for Keap1; TCCCA AATCAGCAGGTATG and TGGCTATGCAACTGACTTCT for GCLM; TGCACATCTACCAACTCC and ATGCCCTATTAGACACCAC for HMOX-1; and ATCCGTAAAGACCTCTATCC and CTCCTGCTTGCTGATCC for -actin.

The PCR reaction system including 1 L of cDNA, 0.4 M of forward and reverse primers, and 10 L of TB Green Premix Ex Taq II, was finally adjusted to the required total volume of 20 L by adding RNase free water. The real-time PCR (Lightcycler 96, Roche, Switzerland) cycling conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10s, 59 °C for 10s, 72 °C for 10 s; (Roche, Switzerland). Following amplification, the melting curves were tested by slowly heating from 60 °C to 95 °C in increments of 0.5°C/s. During this process, we used continuous fluorescence collection to confirm that the peak signal was produced only from the target genes. Relative quantification methods (2^-ΔΔCt) were used to calculate the relative transcriptional level of each gene according to the Cycle threshold value (Ct), in which the expression of Nrf2 and Keap1 genes was normalized against that of -actin.

2.6. Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 22 (IBM, Armonk, NY, USA). The values obtained from biological samples were analyzed using a one-way ANOVA, followed by multiple comparisons using post hoc Tukey test. The homogeneity among different groups was analyzed; if heterogeneity presented among the different groups, the Games–Howell model was utilized. Results are expressed as mean ± standard deviation (SD) for the eight mice in each group (n = 8), or exhibited as Box and Whisker plots, and values of p < 0.05 considered as statistically significant.

3. Results

3.1. Antioxidant Enzymes in Plasma or Muscle

Supplementation of ASX decreased plasma and skeletal muscle GPx and CAT, but increased plasma SOD (Figure 2). It is suggested that supplementation of 15 mg and 30 mg/kg BW of ASX decreased the plasma GPx activity in swimming training mice, compared with that in the SWC groups
(p < 0.001 and p = 0.013, respectively) and SEC group (p < 0.001), However, the dose of 5 mg/kg BW seemed not to affect GPx in the training mice, compared with the two higher dose groups (Figure 2A). In addition, the muscle GPx activity of the SWC group was higher than that of the SEC groups (p = 0.005). Meanwhile, the GPx activity in the SA5 and SA15 groups were higher than in the SEC group (p = 0.001, and p < 0.001, respectively), and the activity in the SA30 group was significantly lower than that of the SWC group (p = 0.002), and almost approached the level of the SEC groups (p = 0.711; Figure 2B). In addition, it was observed that the CAT activity in the SWC group was higher than that of the SEC group (p = 0.024), and supplementation of three different doses (5, 15, and 30 mg/kg BW) of ASX decreased CAT level in the training mice, compared with the SWC group (p < 0.001; Figure 2C). Meanwhile, the highest dose (30 mg/kg BW) of ASX significantly inhibited CAT activity, which was lower than that observed in the SEC group (p = 0.014). However, the SOD activity among the different groups demonstrated a gradually increasing trend (Figure 2D). It was suggested that the SOD activity increased in the SA15 and SA30 groups, compared with the SEC and SWC groups (p < 0.05).

![Figure 2](image-url)

**Figure 2.** Effects of astaxanthin on the GPx, CAT, and SOD activity in swimming training mice. (A) and (B) display GPx activity in plasma and gastrocnemius muscle among different groups, respectively. (C) exhibits CAT activity in gastrocnemius muscle among various groups, respectively. (D) shows SOD activity in plasma among different groups, respectively. The symbol * indicates a significant difference at p < 0.05, and ** indicates a significant difference at p < 0.01.

### 3.2. Plasma Malondialdehyde and Creatine Kinase and Muscle Nrf2-Keap1 Transcription

Supplementation of ASX decreased MDA and CK levels in mice plasma or skeletal muscle and inhibited the transcriptional level of Nrf2 in gastrocnemius muscle (Figure 3). Both plasma and gastrocnemius MDA decreased in the SA15 and SA30 groups, compared with the SWC group (p < 0.001) and the SEC group (p < 0.001). However, the group treated with 5 mg/kg BW of ASX was not shown to decrease plasma and gastrocnemius MDA, which were higher than that in the groups administrated with 15 and 30 mg/kg BW of ASX (p < 0.01; Figure 3A,B). Similarly, administration of ASX decreased the CK levels in swimming training mice (Figure 3C). Following multiple comparison, the results showed that the groups supplemented with 5, 15, and 30 mg/kg BW of ASX decreased plasma CK
value, compared with the SWC group \( (p = 0.031, p = 0.001, \text{and } p < 0.001, \text{respectively}) \). Meanwhile, the CK value in the SA15 and the SA30 groups was lower than in the SEC group \( (p = 0.012 \text{ and } p < 0.001, \text{respectively}) \). In addition, the higher dose of ASX exhibited a greater inhibitory effect on CK and the value in SA30 group was even lower than that of the SA5 group \( (p = 0.005) \).

![Figure 3. Effects of astaxanthin on the MDA level, CK activity, and Nrf2-Keap1 factors’ transcriptional level in swimming training mice. (A) and (B) show the amount of MDA in plasma and muscle among different groups, respectively. (C) represents CK activity in plasma among different groups. (D) outlines transcriptional levels of Nrf2 and Keap1 factors among different groups, in which the fold changes are expressed in relation to the mean of the SEC group. Values are expressed as Box-and-Whisker plots, in which the bottom and top of the box present the first and third quartile, respectively; the band inside the box is always the second quartile (the median); lines extending vertically from the boxes (whiskers) stand for the upper and lower extreme (the highest and lowest number in a set of data), and plus signs indicate the mean for each group. The symbol * indicates a significant difference at \( p < 0.05 \), and ** indicates a significant difference at \( p < 0.01 \).](image)

Additionally, supplementation of 15 or 30 mg/kg BW of ASX downregulated the transcriptional level of Nrf2 by 41% and 39% in the gastrocnemius muscle, compared with the SWC group \( (p < 0.001; \text{Figure 3D}) \). Meanwhile, the Keap1 level of the SEC group remained unchanged, compared with the SWC groups \( (p > 0.05) \) but was higher than that of the SEC group \( (p = 0.029, p = 0.034 \text{ and } p = 0.015, \text{respectively}) \).

3.3. mRNA of Nrf2 and Nrf2-Dependent Enzymes in Heart

The mRNA level of Nrf2 in the SWC group elevated by 1.5-fold after exercise, compared with the sedentary group \( (p = 0.036) \). The mRNA levels of Nrf2 in the SA15 and SA30 groups were shown to be decreased, compared with the SWC group \( (p < 0.01; \text{Figure 4A}) \). Accordingly, the transcriptional level of GCLM and GCLC was suggested to be decreased in astaxanthin supplement groups. It was observed that the mRNA levels of GCLM among SA15 and SA30 groups significantly decreased, compared with the SWC group \( (p < 0.01; \text{Figure 4B}) \). Meanwhile, the mRNA levels of GCLC among the SA5,
SA15, and SA30 groups were significantly lower than the SWC and SEC groups \((p < 0.05; \text{Figure 4C})\). Additionally, NQO-1 was inhibited as well after supplementation of astaxanthin. The mRNA level of NQO-among the SA5, SA5, and SA30 groups significantly decreased, compared with the SWC group \((p < 0.01)\), meanwhile, the mRNA level in the SWC group was higher than the SEC group \((p > 0.05; \text{Figure 4D})\). Meanwhile, the transcriptional level of HMOX-1 was significantly increased in the SWC group \((p < 0.05\); Figure 4E). It was also noted that as the ASX dose proportionally increased, the cNOS activity among the different groups demonstrated an increasing trend \((p < 0.001, \text{respectively}; \text{Figure 5A})\). We found that supplementation of ASX affected NOS level in plasma. It was demonstrated that supplementation of 15 and 30 mg/kg BW of ASX increased the tNOS level in swimming training mice, compared with the SEC group \((p = 0.03 \text{ and } p < 0.001, \text{respectively}; \text{Figure 5A})\). It was also noted that as the ASX dose proportionally increased, the cNOS activity among the different groups demonstrated an increasing trend \((p < 0.05, \text{respectively}; \text{Figure 5B})\). We found that only the group supplemented with 30 mg/kg BW of ASX increased cNOS level, compared with the SWC group \((p = 0.007)\). Meanwhile, the cNOS activity in all the three SA groups was higher than in the SEC group \((p = 0.012, p = 0.004, \text{and } p < 0.001, \text{respectively})\). In addition, as the ASX dose increased, the iNOS activity proportionately decreased \((p = 0.035 \text{ and } p = 0.044, \text{respectively})\).
with exercise training decreases the expression of Mn-SOD and GPx in mouse skeletal muscle and is mainly composed of SOD3, which exerts an important protective role in arterial smooth muscle [43]. Previous report showed that SOD3 in alveolar type II pneumocytes was upregulated by TNF-α and INF-γ through activation of nuclear factor Kappa-B (NF-κB). Further, we found that supplementation of 500 mg/kg BW of Vitamin C combined with exercise training decreases the expression of Mn-SOD and GPx in mouse skeletal muscle and significantly inhibited mitochondrial factors, including peroxisome proliferator-activated receptor p53, Nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (MTF-A), and hampered endurance adaptations [16]. Similarly, Meier et al. found that supplementation of an antioxidant cocktail (coenzyme Q10, 1% N-acetyl-cysteine, and vitamin C) inhibited the expression of SOD, GPx, and CAT in skeletal muscle of exercise-trained mice [17]. Our findings suggest that supplementation of 15 or 30 mg/kg BW of ASX in mice performing the moderate-intensity exercise significantly decreases in GPx and CAT activity in plasma or muscle. Furthermore, we found that 15 and 30 mg/kg BW of ASX also decreased Nrf2 transcription in skeletal muscle, but keap1 remained unchanged. This suggests that the expression of antioxidant enzymes is perhaps suppressed partially via downregulation of the transcription of Nrf2. Because Nrf2 upregulates the expression of antioxidant enzyme genes by acting on antioxidant response element (ARE) and keap1 anchors Nrf2, which will be ubiquitinated and then decomposed [40,41]. Meanwhile, we also observed that heart Nrf2 is downregulated in ASX-administrated groups. Moreover, Nrf2-dependent genes GCLC and GCLM, which are the first-rate limiting enzymes of glutathione synthesis, were also downregulated. Two studies showed that ASX supplementation increased the proportion of glutathione (GSH) content in mice soleus muscle, which was considered as basal storage of antioxidative capacity [29,42]. Although we did not quantify GSH in our study, we observed that mRNA of GCLC and GCLM in heart decreased in the ASX-treated groups. The mRNA levels of both NQO-1 and HMOX-1 also decreased in heart among the ASX-administrated groups in comparison to the SWC group, suggesting the amount of quinones and hemes generated in heart are not necessary to trigger the higher-level expression of NQO-1 and HMOX-1.

In addition, plasma SOD activity increased in the medium and high ASX dosage groups. The plasma SOD is mainly composed of SOD3 (EC-SOD), which exerts an important protective role in the vascular wall, and it was documented that the vasoactive factors such as histamine, vasopressin, oxytocin, endothelin-1, serotonin, and heparin markedly increased the enzyme level in cultured arterial smooth muscle [43]. Previous report showed that SOD3 in alveolar type II pneumocytes was suppressed partially via downregulation of the transcription of Nrf2. Because Nrf2 upregulates the expression of antioxidant enzyme genes by acting on antioxidant response element (ARE) and keap1 anchors Nrf2, which will be ubiquitinated and then decomposed [40,41]. Meanwhile, we also observed that heart Nrf2 is downregulated in ASX-administrated groups. Moreover, Nrf2-dependent genes GCLC and GCLM, which are the first-rate limiting enzymes of glutathione synthesis, were also downregulated. Two studies showed that ASX supplementation increased the proportion of glutathione (GSH) content in mice soleus muscle, which was considered as basal storage of antioxidative capacity [29,42]. Although we did not quantify GSH in our study, we observed that mRNA of GCLC and GCLM in heart decreased in the ASX-treated groups. The mRNA levels of both NQO-1 and HMOX-1 also decreased in heart among the ASX-administrated groups in comparison to the SWC group, suggesting the amount of quinones and hemes generated in heart are not necessary to trigger the higher-level expression of NQO-1 and HMOX-1.
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upregulated by TNF-α and INF-γ through activation of nuclear factor Kappa-B (NF-κB). Further, exercise training increases nitric oxide in mouse vessel endothelial cells, which in turn upregulates expression of SOD3 in adjacent smooth muscle cells [44]. Meanwhile, ASX did not directly scavenge superoxide anion free radicals [24]. The elevated concentration of SOD3 prevents the degradation of NO by oxygen radicals. The nitric oxide system is a fundamental determinant of cardiovascular homeostasis and regulates systemic blood pressure, vascular remodeling, and angiogenesis [45]. We found the plasma constitutive NOS (cNOS) activity was increased in the SA30 group. The cNOS consists of two isoforms: epithelial NOS (eNOS) and neuronal NOS (nNOS) [46], but in the plasma, it is mainly composed of eNOS that are produced by epithelial cells [47]. Moncada et al., reported that nitric oxide (NO) is an endothelium-derived relaxing factor, which contributed to the beneficial effects of exercise on the cardiovascular system [48]. Furthermore, Sessa et al., has shown that chronic exercise in dogs increased coronary vascular nitric oxide production and eNOS expression [49]. Other studies in rats and humans have shown that nitric oxide contributes to glucose uptake and increased skeletal muscle basal glucose disposal [50]. From the above studies, and in line with our data, we concur that ASX can potentially stimulate eNOS activity, and this may facilitate vascular relaxation or indeed contribute to improved recovery after chronic exercise. The increase in plasma iNOS can also be considered as a marker of stimulated inflammatory status [51]. We observed a decrease in iNOS following ASX supplementation in the swimming trained mice. Lee et al., revealed that supplementation of ASX can prevent iNOS expression by blocking expression of proinflammatory genes along with NF-κB [52].

Malondialdehyde (MDA) mainly results from lipid peroxidation of polyunsaturated fatty acids [53]. We observed that administration of 15 and 30 mg/kg BW of ASX significantly decreased plasma or muscle MDA level but there were no significant effects on MDA level following the 5 mg/kg BW dose. Other studies have shown that ASX supplementation can decrease MDA level in addition to blunting antioxidant enzyme activity [29,42,54]. However, there were no substantial changes in MDA in both the placebo and ASX groups in elite young soccer players after 90 days of supplementation [55].

The efflux of muscle CK is an indication of a change to normal membrane structure possibly induced by muscle damage; this increased membrane permeability results in CK leakage into the extracellular space [56]. A previous study suggested that chronic exercise increased CK level in mice plasma [57]. In our study, we found that the CK activity agrees with the profile recorded for MDA. Supplementation of three different dosages of ASX significantly decreased plasma CK, compared with the swimming control group. Other studies provide evidence that ASX can decrease CK activity in mice [26,57] or in humans [55,58]. Nevertheless, Richard et al., reported that supplementation of 4 mg/day ASX in humans did not favorably affect CK values associated with skeletal muscle injury following eccentric resistance strength training [59]. Klinkenberg et al., demonstrated that supplementation of 20 mg/day ASX did not significantly decrease plasma CK in well-trained cyclists [60]. ASX is well defined as an efficient scavenger of ROO• and RO• radicals, which are reactive promoters of lipid peroxidation [24]. Thus, ASX has the potential to inhibit lipid peroxidation on the cell membrane and decrease oxidative stress following chronic exercise (as partially observed by the reduction in MDA in our study).

In summary, 5 mg/kg BW of ASX supplementation did not reduce GPx and CAT activity, nor did it suppress expression of Nrf2 in exercise trained mice. However, as the ASX supplemental dosages were raised to 15 mg/kg BW and 30 mg/kg BW, it seemed to decrease the GPx and CAT level as well as Nrf2 in the moderate-intensity swimming training mice. Therefore, using higher dosages of ASX in moderate-intensity exercise may blunt the expression of antioxidant enzymes. However, in high-intensity exercise, cells can generate an overabundance of RONS, and this may require a higher production of antioxidants to neutralize and balance oxidative stress. This may be a potential reason why there is a dosage-dependent relationship between duration time and ASX supplementation in the exhaustive training mice [28]. In our view, the proper dosage used in exercise may optimize the training effects by protecting against exercise-induced RONS overproduction; however, overdosed
supplementation may impair the beneficial effects from exercise. Currently, the absorptivity of astaxanthin between humans and mice is not widely studied, thus we cannot estimate the relative amount for humans. The safe dosage used for human consumption has been discussed in a previous review, and no adverse effects have been observed when humans were supplemented with dosages ranging from 2.38 to 40 mg/day [61]. Nevertheless, the optimal dosage of ASX supplementation in accordance with different oxidative stress for human health benefits needs further investigation in the future. Finally, there are limitations of this study. Firstly, the physiological status of the exercising animals was not measured due to a lack of appropriate equipment. Secondly, the effects of ASX on the mice only receiving ASX treatment were not evaluated. Moreover, due to financial restrictions, we did not collect a complete set of redox biology indices in tissue or plasma. Lastly, since antioxidant enzymes are predominantly present and most active in the intracellular environment (i.e., CuZnSOD, MnSOD), there may be a differential concentration between sites of determination (i.e., intracellular vs. extracellular), and it is conceivable that the analytical kits have limitations in accurate plasma and muscle quantification. Therefore, in any future investigation, protein levels of selected antioxidant enzymes should be included to further evaluate the effects of ASX on exercise.

5. Conclusions

The findings from this study indicate for the first time that high dosage of astaxanthin suppresses GPx and CAT activity in plasma or muscle in moderate-intensity training mice, and downregulates the transcription of Nrf2 and Nrf2-dependent enzymes in skeletal muslce or heart, along with attenuating plasma and muscle MDA.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6643/11/6/1244/s1, S1: The mouse feed composition used in this study.

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