Protective effects of astaxanthin on a combination of D-galactose and jet lag-induced aging model in mice

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Abstract. Oxidative stress caused free radical and mitochondrial damage plays a critical role in the progression of aging and age-related damage at the cellular and tissue levels. Antioxidant supplementation has received growing attention and the effects of antioxidant on aging are increasingly assessed in both animal and human studies. However, additional and more promising treatments that contribute to the expansion of anti-aging therapies are needed. Astaxanthin, a super antioxidant carotenoid and free radical scavenger, inhibits lipid peroxidation more potently than vitamin E. In the present study, we investigated the preventative effects of astaxanthin on aging using an accelerated aging model: mice chronically treated with a combination of D-galactose and jet lag. After 6 weeks of treatment, astaxanthin administration tended to protect the liver weight loss in aged mice. It is probably by upregulating the mRNA expression of galactose-1-phosphate uridyltransferase, which contribute to the enhancement of D-galactose metabolism. Astaxanthin supplementation also improved muscle endurance of aged mice in a swimming test. These results were associated with reduced oxidative stress in serum and increased anti-oxidative enzymes activities and mRNA expression in vivo. Moreover, astaxanthin reversed the dysregulation of aging-related gene expression caused by the combination of D-galactose and jet lag in the liver and kidney of mice. In conclusion, astaxanthin prevents liver weight loss, ameliorates locomotive muscular function, exerts significant anti-aging effects by reducing oxidative stress and improving the expression of age-related genes in D-galactose and jet lag-induced aging model.

Key words: Aging, D-galactose, Jet lag, Astaxanthin, Antioxidant

THE DRAMATIC FERTILITY DECLINE and improved longevity over the past decades are causing world’s population to aging at one of the fastest rates ever recorded. This change is accompanied by an increase in the prevalence of chronic disease, cognitive decline and disability in the aged population [1]. Denham Harman first proposed the free radical theory of aging in the 1950s [2], and in the 1970s extended the idea to implicate mitochondrial production of reactive oxygen species (ROS) [3]. After that numerous theories were proposed to clarify the mechanism of aging, such as mitochondrial DNA damage, cross linkage theory, biological membrane damage, genetic program theory [4]. Among all these, oxidative stress caused free radical and mitochondrial damage is still postulated to be a major causal factor of senescence [5]. On the other hand, because of the complications and limitation of the use of humans in aging research, various animal models have been developed, which contribute to the better understanding of aging process and the development of new anti-aging therapies [6]. In our previous study, we successfully established an aging model with a combination exposure of D-galactose (D-gal) with jet lag that replicated the natural aging process, and found that abnormal metabolism of D-gal and rhythm disturbance induced the decrease of total antioxidant capabilities in mitochondria and antioxidant gene expression in peripheral tissues, as well as dysregulation of aging-related gene expression [7].

Although oxidative stress is pivotal for the progression of aging, no standard therapy for aging has been established. Many agents and methods have been tested for the management of anti-aging, including caloric restriction...
[8], hormonal therapies [9], telomere-based therapies [10], stem cell therapies [11]. Among these anti-aging strategies, the effects of antioxidant supplementation have been widely addressed by many studies both in human and animal models [12]. Studies have found that chronic vitamin C administration decreased isoprostanes levels in rats [13]. Vitamin C reduces the radicals of α-tocopheroxyl in membranes or low-density lipoprotein (LDL) and thereby inhibits α-tocopheroxyl radical-mediated propagation [12]. Vitamin E, another potent antioxidant, protects against oxidative damage by acting directly with a variety of oxygen radicals. The mechanism of antioxidants may involve neutralizing free radicals, reducing the concentration of peroxide, repairing oxidized membranes, quenching iron to decrease ROS production [14]. Therefore, antioxidants are thought to induce antioxidant gene expression, protect LDL cholesterol from oxidation and provide anti-apoptotic protection of the liver, brain and heart [15]. However, additional and more effective paradigms for testing therapeutics aimed at slowing aging are needed.

The plasma levels of carotenoids, which are lipid-soluble antioxidants, are negatively correlated with aging related diseases, such as inflammation, atherosclerosis, cardiovascular disease, and even mortality, and positively correlated with physical performance [12]. Carotenoids supplementation has been found to improve antioxidant status and reduce lipid peroxidation in human [16]. Astaxanthin, a xanthophyll carotenoid, is abundant in marine animals such as salmon, crab, shrimp, and microalgae such as Haematococcus pluvialis [17]. Astaxanthin has been reported to be 100–500 times stronger than vitamin E in inhibiting lipid peroxidation in vitro [18]. The administration of astaxanthin has been found to ameliorate aging-associated diseases, including inflammation, neurodegeneration, cardiovascular disease, nonalcoholic fatty liver disease [19, 20]. Astaxanthin treatment extends life span of Caenorhabditis elegans through the insulin/insulin-like growth factor 1 (IGF-1) signaling [21]. In addition, the anti-aging effects of astaxanthin in Drosophila melanogaster and mice were associated with decreased oxidative stress [22, 23]. Astaxanthin alleviates brain aging by restoring brain derived neurotrophic factor (BDNF) levels in both the brains and hippocampus in rats [24].

Therefore, we hypothesized that the administration of astaxanthin would inhibit the progression of aging by suppressing aging-induced oxidative stress and the subsequent physical exhaustion and dysregulation of aging-related gene expressions. Here, the preventative effects of astaxanthin in a combination of D-gal and jet lag-induced aging model in mice were investigated. The data revealed that astaxanthin prevented the acceleration of aging process by reducing oxidative stress and improving the mRNA expression of aging-related genes.

Materials and Methods

Animals and experimental design

Eighteen-week-old male C57BL/6 mice were purchased from the China National Laboratory Animal Resource Center (Shanghai, China). They were kept in our animal facilities (illumination with strip lights of 200 lux at cage level; 22 ± 1°C) and maintained on a 12/12-h light/dark cycle. Water and food were available ad libitum. All mice were fed with basal diet (BD) for 1 week to adapt the environment before experiments. The composition of BD was described as previously [25], and the mineral mix and the vitamin mix were prepared according to AIN-76 [26]. To determine the anti-aging effects of astaxanthin, mice were randomly divided into three groups and kept for 6 weeks as follows: (1) control group (Con, n = 8), fed with BD, maintained in normal light/dark cycle and accepted only vehicle (0.9% saline) injection; (2) aging group (DL, n = 8), fed with BD and treated with a combination of daily sterile D-galactose (500 mg/kg BW, Amresco. Solon, OH) injection and jet lag; (3) treating group (DL + AS, n = 8), fed with BD containing 0.01% astaxanthin (American Life Science & Food Inc. Hawthorne, CA) and treated with the combination of D-galactose injection and jet lag. The jet lag carried out by a 12 h-reversal of the light/dark cycle once every 3 days was described as previously [7].

The animals were sacrificed on the last day of the treatment in a normal light/dark cycle. Blood samples were collected, centrifuged at 6,000 g for 5 min at 4°C, and sera were stored at −80°C before use. Mice liver and other tissues were removed, weighed and immediately frozen in liquid nitrogen and kept at −80°C until use. All experiments were performed according to institutional guidelines, and the study was approved by the Research Committee of Zhejiang University of Technology.

Swimming test

The swimming test was performed to examine the locomotive muscular function and anti-fatigue capability 1 week before sacrifice. Mice were put in individual
buckets (29 cm in height and 30 cm in diameter) with 25 cm (depth) of water, and their behavior activity was monitored using an online PC computer equipped with CompACT AMS (Activity Monitoring System, Muromachi, Tokyo, Japan), collecting data every 1 min for total 7 minutes.

**Measurement of antioxidant enzyme activities and MDA level**

The serum was directly used for analyzing. The activities of superoxide dismutase (SOD, CV = 1.7%), catalase (CAT, CV = 1.7%) and glutathione peroxidase (GSH-Px, CV = 3.56%) and the level of malonaldehyde (MDA, CV = 2.3%) in serum were determined using commercial kits (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer’s instructions.

**RT-qPCR analysis**

Total RNA of tissues was isolated with TRIzol reagent (Invitrogen, USA) according to the manufacturer’s protocol. The extracted total RNA was reverse-transcribed into cDNA using an M-MLV reverse transcriptase kit (Takara Biochemicals, Dalian, China). Real-time PCR was carried out using a SYBR® ExScript™ PCR Kit (Takara Biochemicals, Dalian, China). PCR amplification and quantification were performed in a real-time PCR system (ABI-7300, Foster City, CA, USA). The relative quantification of gene expression was analyzed from the measured threshold cycles (Ct) by using the 2−ΔΔCt method in the experiment [27]. Primer sequences of the target genes are listed in Supplementary Table 1. The transcript of the constitutive gene Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a housekeeping gene for data normalization.

**Statistical analysis**

Data were given as mean ± SEM and were analyzed by Student’s t test and one-way ANOVA using Stat View 5.0 program (SAS Institute Inc., Cary, NC, USA). Each single values were considered statistically significant when p values were less than 0.05 (*p < 0.05, **p < 0.01, vs. Con group; *p < 0.05, **p < 0.01, vs. DL group). The anti-fatigue capability of astaxanthin was analyzed by two-way ANOVA followed by Student-Newman-Keuls test.

**Results**

**Astaxanthin ameliorated liver weight loss of aged mice**

We have previously found that the combination of chronic administration of D-gal and jet lag had synergistic effect on the aging process [7], the study design of this aging model was shown in Fig. 1A. To assess the effect of astaxanthin on aging, D-gal and jet lag-induced aging mice were fed with BD containing 0.01% astaxanthin. After 6 weeks of treatment, mice body weights were not affected by either the combination D-gal and Jet lag or astaxanthin (Fig. 1B), suggesting astaxanthin was well tolerated in mice. However, D-gal and Jet lag treatment resulted in significant liver weight loss, and astaxanthin administration tended to increase liver weight ratios, leading levels close to that of control mice (Fig. 1C). On the other hand, excessive exogenous D-gal caused a significant down-regulation of the transcript level of galactose-1-phosphate uridytransferase (Galt), while astaxanthin treatment markedly increased the expression of Galt (Fig. 1D). These results indicated that liver weight loss occurred during the process of aging, and astaxanthin would protect such exacerbation.

**Astaxanthin improved locomotive muscular function in aged mice**

Next, the effects of astaxanthin on locomotive muscular function were examined followed by a swimming test (Fig. 2). Mice received the induction of D-gal and jet lag lost their physical endurance after 6 min and 7 min, compared with that of control group (two-way ANOVA, p < 0.01). This loss of locomotive muscular function was recovered by the administration of astaxanthin (Fig. 2), suggesting that the anti-fatigue capability of astaxanthin might contribute to the postponing of aging process.

**Astaxanthin protected against oxidative stress during the process of aging**

Oxidative stress involved in critical aspects of the aging process and contributes to impaired physiological function. Therefore, we next evaluated the oxidative stress by measuring MDA levels and the main anti-oxidative enzymes activities in serum (Fig. 3). The results obtained here indicated that the activities of CAT, GSH-Px and SOD were all decreased in D-gal and jet lag induced aged mice, whereas astaxanthin administration increased these anti-oxidative enzymes activities significantly (Fig. 3A–C). The levels of MDA, an index of lipid peroxide and oxidative stress, in the serum were
increased markedly by the D-gal and jet lag treatment, revealing exaggerated oxidative stress in aged mice. Astaxanthin treatment resulted in less oxidative stress (Fig. 3D). Thus, the anti-aging effect of astaxanthin is associated with reduced oxidative stress and increased anti-oxidative enzymes activities in mice.

To further investigate the effect of astaxanthin on oxidative stress, the mRNA expressions of antioxidant genes in the liver and kidney were assessed. The expressions of antioxidant genes including Cat, Gpx1, Sod1 and Sod2 in the liver were decreased by the combination of D-gal and jet lag induction, while they were increased by astaxanthin administration (Fig. 4A). Similar results were found in the kidney (Fig. 4B). These data suggested that anti-oxidative effects of astaxanthin in aged mice were associated with the increase of antioxidant genes expression.

**Astaxanthin improved the dysregulation of aging-associated genes expression**

Finally, the anti-aging effects of astaxanthin were clarified by the mRNA expression of aging-related genes. The expression of *sirtuin 1* (*Sirt1*), which plays a critical role in the regulation of aging and longevity in mammals, was decreased both in the liver and kidney of D-gal and jet lag treated mice (Fig. 5). In contrast, β-D-galactosidase 1 (*Glb1*), a novel senescence marker, was increased in the liver and kidney of aged mice (Fig. 5). Astaxanthin administration reversed the expression pattern of *Sirt1* and *Glb1* (Fig. 5). Moreover, the mRNA expression of *Klotho*, identified as an “aging-suppressors” gene, was down-regulated in the kidney of aged mice, suggesting the acceleration of aging process by the com-

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**Fig. 1** Astaxanthin ameliorated liver weight lost in aged mice. (A) Experimental design of D-galactose combined with jet lag-induced aging model. After 1 week of adaption, mice were received a daily sterile D-galactose (500 mg/kg BW) injection, and light/dark cycle was reversed by extending the light period for 24 h every 3 days to induce chronic jet lag. The white/black bar represents the light phase and dark phase of 12/12-h light/dark cycle. The experiment was carried out for 6 weeks, and mice were sacrificed on the last day of the treatment in a normal light/dark cycle. (B) Mice body weights at the end of the experiments. (C) Liver weight ratios of mice. (D) mRNA expression of *galactose-1-phosphate uridylytransferase* (*Galt*) in the liver of mice. n = 8, *p < 0.05, **p < 0.01, vs. Con group, #p < 0.05, ##p < 0.01, vs. DL group.

**Fig. 2** Astaxanthin improved locomotive muscular function in aged mice. Swimming tests were carried out after 5 weeks of treatment, and mice behavior activity was monitored using an online PC computer by collecting data every 1 min for 7 min. n = 8, *p < 0.05, **p < 0.01, vs. Con group, #p < 0.05, ##p < 0.01, vs. DL group.
Fig. 3  Astaxanthin protected against oxidative stress in serum. (A) Serum catalase (CAT) activities. (B) Serum glutathione peroxidase (GSH-Px) activities. (C) Serum superoxide dismutase (SOD) activities. (D) Serum malonaldehyde (MDA) levels. \( n = 8, \ast p < 0.05, \ast\ast p < 0.01, \) vs. Con group, \#\#p < 0.01, vs. DL group.

Fig. 4  Astaxanthin increased the expression of antioxidant genes in the liver and kidney. RT-qPCR analysis of mRNA expression of Cat, Gpx1, Sod1, Sod2 in the liver (A) and kidney (B) after 6 weeks of treatment. \( n = 8, \ast p < 0.05, \ast\ast p < 0.01, \) vs. Con group, \#\#p < 0.01, vs. DL group.
Combination of D-gal and jet lag (Fig. 5B). Astaxanthin treatment inhibited the decrease of Klotho, leading to the levels close to that of control mice. Therefore, astaxanthin exert anti-aging effect by improving the dysregulation of expression of aging-related genes.

Discussion

This study investigated the effects of the potent antioxidant carotenoid astaxanthin on aging and elucidated the potential mechanism underlying the effects. We found that astaxanthin exhibited significant preventative effects on D-gal and jet lag-induced aging model. Astaxanthin attenuated liver weight loss and downregulation of Galt expression in the liver, improved locomotive muscular function in a swimming test, enhanced both serum anti-oxidative enzymes activities and antioxidant genes expression in vivo. In addition, astaxanthin ameliorated the dysregulation of aging-related genes expression both in the liver and kidney of aging mice.

Aging is the progressive loss of tissue or cellular functions that increases the probability of promoting degenerative pathologies [28]. Various theories have been proposed to explain the ageing process, but none has yet been generally accepted. On the other hand, the initial free radical, which related to the basic aging process [2], is accepted by more and more gerontologists as a possible explanation of the chemical reactions at the basis of aging [12]. Originally, increased reactive oxygen species (ROS) generation, or oxidative stress caused by mitochondria damage, plays an important role in the acceleration of aging process [29, 30]. Therefore, inhibiting ROS production by mitochondria-targeted rechargeable antioxidants provided the basic approach to prevent age-related disorders [31]. The establishment of aging model plays a pivotal role in the aging related research. However, the natural aging models are difficult to be used in aging-related research because of the time limitation. D-gal-induced aging model showed decreased activity of antioxidant enzymes, dysfunctional mitochondria, neurotoxicity, poor learning and memory, low immune response and a shortened lifespan [32]. These characteristics resemble those of the natural aging process in human. Thereby, D-gal-induced aging mode is widely used for the investigation of aging mechanism and screening of anti-aging agents. On the other hand, chronic rhythm disturbance of organisms will accelerate the aging process [33]. Consistently, we have found that
the combination of D-gal and jet lag resulted in the acceleration of aging process in our previous study [7]. In addition, jet lag, shift working, late night eating, light pollution are becoming more and more prevalence in the modern society, which will lead to the disruption of circadian rhythm. Therefore, the natural aging process is somehow also accompanied with circadian disorder, and the combination of D-gal and jet lag induced aging model is more suitable for the current study.

Consistent with previous studies, D-gal-induced acceleration of aging process was associated with increased oxidative stress, decreased antioxidant enzyme activities (Figs. 3 and 4) [34-36]. Dietary carotenoids are thought to provide health benefits by decreasing the risk of aging associated diseases, and the beneficial effects of carotenoids are thought to be due to their role as antioxidants [37]. Astaxanthin, with super antioxidative capability, increased the activities of antioxidant enzyme and decrease the oxidative stress marker MDA levels in serum (Fig. 3), thereby exerting anti-aging effect in experimental aging model. Moreover, consisting with previous studies [38, 39], supplementation with astaxanthin caused an upregulation of antioxidant genes expression in peripheral tissues, such as liver and kidney (Fig. 4), which contribute to the protection against oxidative stress. On the other hand, the nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) controlled the expression of antioxidant genes via upstream antioxidant response element (ARE) regions [40]. Recent studies have found that astaxanthin activated Nrf2-ARE pathway to suppress oxidative stress and thereby exert protective effects [41, 42]. Therefore, the upregulation of antioxidant genes by astaxanthin might be induced by the activation of Nrf2-ARE pathway in our study. Further studies are needed to examine the effects of astaxanthin on Nrf2−/− mice, which would contribute to the better understanding of the molecular mechanism of astaxanthin in aging.

The liver is the key organ for metabolism and detoxification, and numerous age-associated changes in liver structure and function have been found. Its function gradually decline due to age-associated structural atrophy [43]. Moreover, recent studies indicated that aging was associated with a 24% reduction of liver weight in males and an 18% reduction in females [44]. The present results indicated that the liver was atrophied slightly in D-gal and jet lag-induced aging mice (Fig. 1C), and astaxanthin treatment tended to increase the liver weight ratios to protect against function decline. In addition, D-gal was mainly metabolized in the liver, and chronic D-gal administration would result in toxicity, which was accompanied by downregulation of Galt expression in the liver [45]. Astaxanthin increased the mRNA expression of Galt (Fig. 1D), thereby enhanced the metabolism and usage of D-gal, partly contributing to the protection of liver function and weight loss in aging mice.

Skeletal muscle is another major tissues affected by aging, and the atrophy of skeletal muscle is a highly prevalent as the rapid increase in the number of aged people [46]. The locomotive system is fundamental in maintaining the quality of life of the aged people, which directly affects the basic activities of daily life [47]. Previous studies have found that D-gal-induced aging models replicated the pathophysiological features of human aging, including skeletal muscle atrophy [48, 49]. Similarly, combined D-gal and jet lag treatment caused a quick decline of physical endurance in aged mice in our study (Fig. 2). On the other hand, a randomized, double-blind study has found that intake of astaxanthin improved muscle endurance in human [17]. Astaxanthin supplementation increased swimming time significantly before exhaustion compared with control mice, which further support the effect of astaxanthin on muscle endurance [50]. The effect of astaxanthin on delaying physical exhaustion may involve the redox imbalance in both serum and muscle [51]. Moreover, multiple studies have demonstrated that astaxanthin improved muscular function by inhibiting oxidative stress in the muscle, such as reduce ROS production, increase antioxidant genes expression and anti-oxidative enzymes activities, and decrease mitochondria damage [51-53]. Therefore, astaxanthin administration significantly restored the loss of muscular function in our study (Fig. 2), probably by reducing oxidative stress in mice. Further studies focus on the pathological analysis of muscle, oxidative state and pathways involved in oxidative stress in skeletal muscles will provide new insight into the mechanism of astaxanthin on muscular function.

Silent information regulator (Sir) 2, also known as “sirtuins”, mediates lifespan extension in model organisms and prevents apoptosis in mammalian cells [54]. Sir1 may increase organismal longevity by tipping FOXO-dependent responses away from apoptosis and toward stress resistance [54]. Studies have found that Sir1 expressions in the liver, kidney were decreased in D-gal-treated mice [55]. In addition, Glb1, which coding β-galactosidase, has been reported to increase during the replicative senescence of fibroblast cultures and has been
used widely as a marker of cellular senescence in vivo and in vitro [56]. It has been known that Klotho+/– mice exhibit extremely shortened life span with multiple disorders resembling human, including atherosclerosis, osteoporosis, emphysema, and infertility [57]. Moreover, the expression of Klotho gene was found to be decreased in the liver and kidney during aging process [58, 59]. Consistently, our results indicated that these aging-related gene expressions were also dysregulated in the liver and kidney of aged mice (Fig. 5), suggesting that the aging model was established successfully. Therefore, the anti-aging effect of astaxanthin may also partly through regulation of these genes. Further investigations, such as senescence-associated beta-galactosidase (SAβG) staining and protein analysis of SIRT1 and KLOTHO, will provide more details about the tissue damage levels post D-gal and jet-lag challenges, and how asaxanthin exerts its anti-aging effects on this aging model.

In summary, this study demonstrated that astaxanthin, a potent antioxidant carotenoid, inhibited D-gal and jet lag-induced the progression of aging by enhancing hepatic D-gal metabolism, improving muscular function and preventing liver weight loss. The beneficial effects of astaxanthin were attributable in part to decreased oxidative stress in the serum and peripheral tissues, as well as the improvement of dysregulation of aging-related gene expression. Further studies focusing on the therapeutic effects and detailed molecular mechanism would contribute to a better understanding of the novel action of astaxanthin on aging and aging-related diseases. Taken together, astaxanthin might be a novel and promising anti-aging agent.

**Conflicts of Interest**

All authors report no conflicts of interest.

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