# Astaxanthin promotes mitochondrial biogenesis and antioxidant capacity in chronic high-intensity interval training

European Journal of Nutrition

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--Manuscript Draft--

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| **Manuscript Number:** | EJON-D-22-00316R1 |
| **Full Title:** | Astaxanthin promotes mitochondrial biogenesis and antioxidant capacity in chronic high-intensity interval training |
| **Article Type:** | Original Contribution |
| **Keywords:** | Astaxanthin; PGC-1α; RONS; Nrf2; Mitochondrial biogenesis; Oxidative stress; High-intensity interval training |
| **Corresponding Author:** | Yingsong Zhou, Ph.D<br>Ningbo University<br>CHINA |
| **Corresponding Author Secondary Information:** | |
| **Corresponding Author's Institution:** | Ningbo University |
| **Corresponding Author's Secondary Institution:** | |
| **First Author:** | Yang Wang, M.D. |
| **First Author Secondary Information:** | |
| **Order of Authors:** | Yang Wang, M.D. |
| | Xiaoping Chen, Ph.D |
| | Julien S. Baker, Ph.D |
| | Gareth W. Davison, Ph.D |
| | Shujun Xu, Ph.D |
| | Yingsong Zhou, Ph.D |
| | Xiaoming Bao, Ph.D |
| **Order of Authors Secondary Information:** | |
| **Funding Information:** | Natural Science Foundation of Ningbo (2019A610347) — Dr. Yingsong Zhou |
| **Abstract:** | Purpose: Reactive oxygen and nitrogen species are required for exercise-induced molecular adaptations; however, excessive exercise may cause cellular oxidative distress. We postulate that astaxanthin (ASX) can neutralize oxidative distress and stimulate mitochondrial biogenesis in high-intensity exercise-trained mice.<br>Methods: Six-week-old mice (n = 8/group) were treated with ASX (10 mg/kg BW) or placebo. Training groups participated in 30 min/day high-intensity interval training (HIIT) for six weeks. Gastrocnemius muscle was collected and assayed following the exercise training period.<br>Results: Compared to the HIIT control mice, the ASX-treated HIIT mice reduced malonaldehyde levels and upregulated the expression of Nrf2 and FOXO3a. Meanwhile, the genes NQO1 and GCLC, modulated by Nrf2, and SOD2, regulated by FOXO3a, and GPx4, were transcriptionally upregulated in the ASX-treated HIIT group. Meanwhile, the expression of energy sensors, AMPK, SIRT1, and SIRT3, increased in the ASX-treated HIIT group compared to the HIIT control group. Additionally, PGC-1α, regulated by AMPK and SIRT1, was upregulated in the ASX-treated HIIT group. Further, the increased PGC-1α stimulated the transcript of NRF1 and Tfam and mitochondrial proteins IDH2 and ATP50. Finally, the ASX-treated HIIT mice had upregulations in the transcript level of mitochondrial fusion factors, including Mfn1, Mfn2, and OPA1. However, the protein level of AMPK, SIRT1, and FOXO3a, and the transcript level of Nrf2, NQO1, PGC-1α, NRF1, Mfn1, Mfn2, and OPA1 decreased in |

| | |
|---|---|
| | the HIIT control group compared to the sedentary control group.<br>Conclusion: Supplementation with ASX can reduce oxidative stress and promote antioxidant capacity and mitochondrial biogenesis during strenuous HIIT exercise in mice. |
| **Suggested Reviewers:** | Michalis Nikolaidis, ph.D<br>Aristotle University of Thessaloniki School of Sciences: Aristoteleio Panepistemio Thessalonikes Schole Thetikon Epistemon<br>nikolaidis@auth.gr<br>This scientist is an expert in the field of oxidative stress biomarkers, free radical scavengers, and free radical biology and is able to provide an objective assessment of our manuscript. |
| | Tom Clifford, ph.D<br>Loughborough University Loughborough Sports Development Centre: Loughborough University Loughborough Sport<br>T.Clifford@iboro.ac.uk<br>This scientist is an expert in the area of exercise physiology and nutrition and is able to provide an objective assessment of our manuscript. |
| | Melitta McNarry, ph.D<br>Professor, Swansea University College of Engineering<br>m.mcnarry@swansea.ac.uk<br>This scientist is an expert in exercise science and physiology and is able to provide an objective assessment of our manuscript. |
| | Huw Wiltshire,, ph.D<br>Professor, Cardiff Metropolitan University<br>hwiltshire@cardiffmet.ac.uk<br>This scientist is an expert in sports medicine, physical fitness, and exercise physiologyand is able to provide an objective assessment of our manuscript. |

Dear Professor Bruce Arthur Griffin and Reviewer,

Please find the revised manuscript titled "Astaxanthin promotes mitochondrial biogenesis and antioxidant capacity in chronic high-intensity interval training."

Thanks for giving us many valuable suggestions. We have made changes, checked the data, and corrected the errors in the manuscript according to the comments.

1, The total training period was six weeks, previously described as four weeks in the original version. The final HIIT training speed was close to the maximal velocity of the mice tested.

2, We supplemented the two-way ANOVA data in the revised version.

3, We used boxer and whisker plots instead of histogram plots to convey more data details.

The following changes have been listed according to two reviewers, and the changes in the manuscript were colored in blue.

Reviewer #1: Suggestions:

- Better justify the choice of High-intensity interval training for the model studied.

- Justify the chosen dose of astaxanthin supplementation.

- Improve the wording between lines 135 and 136.

Reviewer #2: This paper describes an interesting study examining the effects of astaxanthin consumption in mice undergoing high intensity interval training. My main concern about the paper is that I do not believe the explanation of the data in the results and discussion section really do the

**Commented [zy1]:** It has been enriched in the paragraph of the lines 89-96 and 141-161.

**Commented [zy2]:** We have clarified why the dose was used in this study in the part of method, lines 137-138, and discussion, lines 412-425.

**Commented [zy3]:** These sentences have been improved.

work justice. Both sections could be substantially improved to provide more detail, clarity and critical depth. There are currently numerous minor inconsistencies. I provide a few examples of these below, but this is not an exhaustive list, and the authors should check through both the results and discussion line by line to remove such errors and inconsistencies. The discussion section could also provide a more in-depth evaluation of the data generated. In particular, the genes and proteins analysed are inter-linked and yet the authors do consider in sufficient detail how the findings for one analyse link to those for another.

An additional fundamental point that ought to be considered at some point in the paper is the dose of astaxanthin. Why was 10mg/kg body weight selected and how would this relate to typical nutritional intakes for humans. Could such an intake be achieved through normal diet or is this a nutrition study or is the astaxanthin being used as a pharmaceutical/nutraceutical?

**Commented [zy4]:** It has been clarified in the part of discussion, lines 412-425.

Examples of concerns about presentation of the data

Some elements of the authors' data interpretation strike me as curious. For example, in lines 373-378 the authors state "the transcriptional level of GPx4 in the astaxanthin-supplemented HIIT group was more than twice that of the HIIT control group, while GPx4 in the astaxanthin-supplemented sedentary group was four times higher than the inactive control group; this indicates that astaxanthin can promote GPx4 expression following HIIT. Moreover, astaxanthin stimulates GPx transcription in the absence of an exercise intervention." Isn't a more logical interpretation of these data that the astaxanthin exposure induces GPx4 expression and that the HIIT partially suppresses the induction of GPx4 by astaxanthin?

**Commented [zy5]:** Yes, we have revised this part based on your suggestion.

In lines 385-387, the authors state "The present study shows that excessive high-intensity exercise can impair the SIRT1, SIRT3, and FOXO3a expression, however, supplementation of ASX seems to promote the expression of these genes." Inspection of the data presented in figures 2b and 3a shows that SIRT1 and FOXO3a mRNA levels were indeed apparently reduced in the EC group (exercise without astaxanthin) compared to the SC group (sedentary without astaxanthin) and other groups. However, the data in figure 3c shows that SIRT3 mRNA levels were significantly increased, rather than decreased, in the EC versus the SC group. Moreover, the statistical analyses of the data presented in figures 2b and 3a suggest astaxanthin had no significant effect on SIRT1 or FOXO3a mRNA levels, respectively, while it did appear to increase SIRT3 mRNA levels. Thus, the authors

summary statement does not appear to stand up to scrutiny.

Throughout the results and discussion, the authors have intermittently referred to protein activity. However, their study included semi-quantitative analysis of specific mRNA and protein levels, but they have not performed any protein activity determinations. This is important because, as is explained in the discussion, a variety of post-translational regulatory processes regulate the activity of Nrf2, AMPK, FOXO3a, SOD2 and PGC-1a. Other assays could have been used to gain more insight into the activities of the target proteins. For example, the subcellular distribution of Nrf2 (nuclear versus cytoplasmic) could have been determined or an EMSA assay used to determine activity of nuclear proteins binding to an oligonucleotide containing an antioxidant response element sequence. The authors should replace the term activity wherever it has been used to describe assays of protein quantity. They should also discuss the limitations of not performing any activity analyses in their discussion.

The statement made in the sentence that runs from line 228-229 appears to repeat information in sentence on lines 227-228.

Lines 241-243 are not well phrased. The text initially suggests there was a difference, but the statistical analysis suggests otherwise. Additionally, figure 2a suggests there was a significant difference between AMPK protein concentrations in the SC versus EC groups (p marked as <0.05) but from the data presented it does not look convincing that there would be a statistically significant difference between these groups. Can this be checked? If the significant difference is confirmed, this should be mentioned in the main text.

The sentence on lines 271-273 seems to finish with a direct contraction of what is said in the first part of the sentence.

Lines 279-280 The statement that "NRF1 was lower in the EC than in the SC group ($p<0.001$)" appears to have the wrong p value since the difference is marked ** rather than *** in figure 4c.

Lines 324 and 340 Nrf2 is not an antioxidant enzyme it is a transcription factor.

In lines 324-326 the authors state "NQO-1, and FOXO3a, in the gastrocnemius of the HIIT control group was lower than that of the sedentary group, which may have been caused by the excessive

**Commented [zy6]:** We have checked the data and redrawn the figures.

**Commented [zy7]:** The word “activity” in the text has been changed to quantity or level. And we have supplemented the limitation of this study at the end of the discussion. Lines 542-549.

**Commented [zy8]:** We have checked the data, and corrected some errors.

training load." However, NQO-1 mRNA was also significantly reduced by astaxanthin treatment alone and the authors do not address this point.

327-330 I think the way this sentence is worded is misleading or, at least, easy to misunderstand - it finishes by suggesting that "a two-fold increase in MDA concentration in the astaxanthin supplemented exercise group" is evidence that astaxanthin protected the HIIT group from oxidative distress. The evidence for that is not based on an increase in MDA in the EA group but by a decrease in MDA in the EA group compared with the EC group.

Statistical analysis

The study was designed to evaluate the impact of 2 independent treatments, independently and in combination - high intensity interval training and astaxanthin supplementation. Would it not be more appropriate to use 2-way ANOVA to analyse the data? This approach would have the benefit of additional statistical power and be able to identify significant interactions between the two treatment types of which there seem to be many.

Minor and technical points

Justify the selection of n=8 for the treatment groups (line 129). Was a power calculation performed? Were the numbers chosen based on prior studies?

Lines 98-100 add suitable citation to evidence this mechanism.

Lines 109 and 112 use past tense for the verbs as the work has been completed.

Line 117 Why was olive oil selected as the vehicle. Olive oil may contain non-nutrient antioxidants, particularly if extra-virgin olive oil was selected. As such, this seems a slightly strange choice for an antioxidant study.

Line 142 Do the authors mean "cryopreservation" rather than "cryopreserved"?

Line 153 Please provide a more detail about the SDS-PAGE (e.g. gel composition)

Section starting at line 165 Was that RNA purity and integrity assessed? If so, please provide details. Please also provide a bit more detail about the primers selection/design. Where primers selected to span intron-exon boundaries?

Section starting at line 221. I am nots convinced the title is strictly correct. Measuring nrf2 protein and mRNA is not exactly the same as measuring nrf2 activity. Subcellular fractionation (i.e.

**Commented [zy9]:** It has been improved based on the suggestion.

**Commented [zy10]:** The Two-way ANOVA has been applied, and we have supplemented the content in the results.

**Commented [zy11]:** We have clarified the sample size chosen (line 134-135)

**Commented [zy12]:** The citation has been added, and some sentences have been revised.

**Commented [zy13]:** It has been described in discussion line 423-425, our purpose is to improve the absorption of ASX.

**Commented [zy14]:** Yes, the word “cryopreservation” has taken the place of “cryopreserved”

**Commented [zy15]:** It has been supplemented in the line 167.

**Commented [zy16]:** The RNA purity and integrity were assessed, and the details has been described in the lines 181-187

cytoplasmic versus nuclear) and/or XXX assay would indicate activity.

Line 225 should read "transcript level of Nrf2"

Line 262 should start "Transcript levels of GPx4…"

Line 278 should refer to figure 4c rather than 3c

Line 410-411 Please clarify what is meant by "Exercise-induced PGC-1α can transcribe proximal promoter of PGC-1α, indicating further amplification of the PGC-1α pathway"

We sincerely appreciate the valuable comments on our manuscript, and we look forward to receiving further information from you. If you have any queries, please don't hesitate to contact me.

Best wishes,

Dr. Yingsong Zhou

Faculty of physical education and sport sciences

Email address: zhouyingsong@nbu.edu.cn

Tel: 86-13780054166

Date: 2022/3/8

**Commented [zy17]:** The title has been changed to "**Nrf2 expression and associated downstream genes**" line 229.

**Commented [zy18]:** These sentences have been revised.

**Commented [zy19]:** Thanks for your correction.

**Commented [zy20]:** We have clarified this point in the lines 516-518

# Astaxanthin promotes mitochondrial biogenesis and antioxidant capacity in chronic high-intensity interval training

Yang Wang[1], Xiaoping Chen[1], Julien S. Baker[2], Gareth W. Davison[3], Shujun Xu[4], Yingsong Zhou[1*], Xiaoming Bao[5,6*]

1 Faculty of Physical Education of Sports Science, Ningbo University, Ningbo, Zhejiang, PR China
2 Centre for Health and Exercise Science Research, Hong Kong Baptist University, Kowloon Tong, HK, 999077, China
3 Sport and Exercise Sciences Research Institute, Ulster University, Newtownabbey BT37 0QB, UK.
4 Medical School, Ningbo University, Ningbo, Zhejiang, PR China
5 Department of Cardiology, Hwa Mei Hospital, University of Chinese Academy of Sciences, Ningbo, Zhejiang, PR China.
6 Department of Global Health, Ningbo Institute of Life and Health Industry, University of Chinese Academy of Sciences, Ningbo, Zhejiang, PR China
*Corresponding author: zhouyingsong@nbu.edu.cn (Y. Zhou), 18668271580@163.com (X. Bao).

## Abstract

**Purpose:** Reactive oxygen and nitrogen species are required for exercise-induced molecular adaptations; however, excessive exercise may cause cellular oxidative distress. We postulate that astaxanthin (ASX) can neutralize oxidative distress and stimulate mitochondrial biogenesis in high-intensity exercise-trained mice.

**Methods:** Six-week-old mice ($n$ = 8/group) were treated with ASX (10 mg/kg BW) or placebo. Training groups participated in 30 min/day high-intensity interval training (HIIT) for six weeks. Gastrocnemius muscle was collected and assayed following the exercise training period.

**Results:** Compared to the HIIT control mice, the ASX-treated HIIT mice reduced malonaldehyde levels and upregulated the expression of Nrf2 and FOXO3a. Meanwhile, the genes *NQO1* and *GCLC*, modulated by Nrf2, and *SOD2*, regulated by FOXO3a, and *GPx4*, were transcriptionally upregulated in the ASX-treated HIIT group. Meanwhile, the expression of energy sensors, AMPK, SIRT1, and SIRT3, increased in

the ASX-treated HIIT group compared to the HIIT control group. Additionally, PGC-1α, regulated by AMPK and SIRT1, was upregulated in the ASX-treated HIIT group. Further, the increased PGC-1α stimulated the transcript of *NRF1* and *Tfam* and mitochondrial proteins IDH2 and ATP50. Finally, the ASX-treated HIIT mice had upregulations in the transcript level of mitochondrial fusion factors, including *Mfn1*, *Mfn2*, and *OPA1*. However, the protein level of AMPK, SIRT1, and FOXO3a, and the transcript level of *Nrf2*, *NQO1*, *PGC-1α*, *NRF1*, *Mfn1*, *Mfn2*, and *OPA1* decreased in the HIIT control group compared to the sedentary control group.

**Conclusion:** Supplementation with ASX can reduce oxidative stress and promote antioxidant capacity and mitochondrial biogenesis during strenuous HIIT exercise in mice.

**Keywords:** Astaxanthin; PGC-1α; RONS; Nrf2; Mitochondrial biogenesis; Oxidative stress; High-intensity interval training

## Statements and Declarations

### Conflicts of Interest

The authors declare no conflict of interest.

## Introduction

Exercise leads to a plethora of physiological adaptations, such as mitochondrial biogenesis, upregulation of antioxidant capacity, and an increase in exercise capacity [1, 2]. Exercise-induced reactive oxygen and nitrogen species (RONS) are the primary chemical mediating agents causing animal cell adaptation [2]. The optimum amount of RONS leading to exercise-induced adaptation is termed oxidative eustress, with $H_2O_2$ as the primary functional molecule estimated to be between 10nM~100nM [3]. Supplementation of exogenous antioxidants has recently become controversial [4–6], while previous work from our laboratory has shown that supplementation with high-dose astaxanthin partially hampers the expression of antioxidant-related pathways in moderately intense swimming mice [7]. In addition, many other studies have also demonstrated that antioxidant supplementation inhibits exercise-induced cell adaptations [8–11]. However, antioxidant treatment has also been shown not to hamper physiological adaptation following exercise [12–14]. Astaxanthin (ASX), a xanthophyll carotenoid, with its antioxidant activity quantified as 10-fold greater than other carotenoids, such as β-carotenoids, and 100-fold greater than α-tocopherol (vitamin E) [15], can directly scavenge selective RONS such as peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$), and singlet oxygen in a dose-dependent manner, but how it affects the transcription of antioxidant enzymes is still unclear [16]. Previous work has demonstrated that ASX can prolong exercise duration and inhibit exercise-induced muscle and heart damage in strenuous training mice [17]. In addition, a study using astaxanthin therapy has shown an improvement in mitochondrial function by increasing ATP synthesis, mitochondrial mass, and Cytochrome C oxidoreductase activity [18]. Thus, ASX seems to be a novel supplement for exercise, especially considering its salient antioxidant scavenging ability.

High-intensity interval training (HIIT) is defined as near-maximal exercise generally performed at an intensity that elicits ≥ 80% (but often 85–95%) of maximal heart rate

with multiple short-time cycles [19]. A recent study demonstrated that exercise-induced ROS production in skeletal muscle is intensity-dependent [20]. Thus, high-intensity interval training may lead to a higher rate of RONS generation, causing a greater stimulation of physiological adaptation [21]. We speculated that a long-term excessive amount of HIIT might result in oxidative distress, and administration of ASX will neutralize the HIIT-induced oxidative distress. To investigate this further, we used a long-term HIIT mice model to evaluate the effect of ASX on oxidative stress and cell adaptation.

Mitochondrial biogenesis is one of the most salient mechanisms controlling adaptation to exercise. Moreover, an examination of antioxidant enzyme regulation may also provide insights into the adaptive responses to antioxidant capacity in exercise training. To complement the quantification of PGC-1α (as the primary marker of mitochondrial biogenesis), we further explored associated signaling pathways to provide mechanistic insights related to the effects of ASX on the HIIT-intervened mice. AMP kinase (AMPK) is a metabolic stress and nutrient deprivation sensor. Exercise-induced RONS activates AMPK by oxidizing two localized cysteine sites, cysteine 299 and 304, which subsequently activates PGC-1α [22]. The activation of nuclear respiratory factors 1 and 2 (NRF1/2) and mitochondrial transcription factor A (Tfam) are also involved in this process leading to mitochondrial biogenesis. Secondly, nuclear factor E2-related factor 2 (Nrf2) is a crucial regulator of cellular defense against oxidative stress by regulating the transcription of components of the glutathione and thioredoxin antioxidant systems [23]. In addition, the transcription factor fork-head box protein O3 (FOXO3a) is also an essential regulator of cellular homeostasis, stress response, and longevity. This transcription factor can modulate various stress responses to oxidative stress, hypoxia, and DNA damage [24]. In summary, this study aimed to clarify the role of ASX in mitochondrial biogenesis following long-term HIIT by observing signaling pathways

aligned to PGC-1α. Furthermore, the effects of astaxanthin on endogenous antioxidant capacity as mediated by Nrf2 and FOXO3a will also be examined.

## Materials and methods

### Astaxanthin Source and Animals

Astaxanthin (Cat. No. SML 0982) was purchased from Sigma-Aldrich (Saint-Louis, MO, USA), dissolved in olive oil, and stored at -20°C. Thirty-two male C57BL/6 mice (6 weeks old) with weights ranging from 20~25g were purchased from Shanghai Slac Laboratory Animal Co. Ltd., 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 the mouse feed is listed in the Supplementary material section.

### Exercise Protocol

The mice were randomly divided into four groups, consisting of a sedentary control (SC) group, a sedentary plus ASX (SA) group, an exercising control (EC) group, and an exercising plus ASX (EA) group; each group comprised eight mice. The sample size ($n$ = 8) of each group used was based on the previous studies [7, 25, 26]. The SA and EA mice groups were orally treated with 0.1 ml of the ASX and olive oil mixture (10 mg/kg Body Weight (BW) ASX supplementation). The supplemented ASX quantity selected for administration was based on previously published works [7, 25–27]. The SC and EC groups were orally administered 0.1 mL of olive oil by gavage each day. All mice were fed the ASX mixture or olive oil two hours before exercise.

The Maximal intensity running capacity test was carried out as previously described [20]. Briefly, mice were acclimatized to the treadmill (10 min at 9.6 m/min) three times a week prior to completing the maximal running test. The maximal running test

commenced at 9.6 m/min for 5 min with a horizontal inclination, followed by a stepwise increase (0.2 m/s) in running speed until exhaustion. The mice that fell back on the electric grid three times within 30 s were defined as being exhausted. The maximal running rate was determined as the last completed stage during the incremental test.

High-intensity interval training (corresponding to nearly 100% maximum mice running speed) was performed on an animal treadmill with an electric grid equipped at the end of the treadmill. The rate of treadmill running was initiated by 14 meters/min, increasing by 2 m/min each day until the maximal speed was reached. The interval training program was performed with an alternate of 2 minutes running and 1 minute of rest, ten sets each time, five times a week for six weeks. After six weeks of training, all experimental mice were anesthetized by intraperitoneally injecting 0.3 mL/kg chloral hydrate (10%) and sacrificed four hours postexercise. Gastrocnemius muscles were quickly isolated and packed in a cryopreservation tube and stored at -80 °C for analysis. The experimental protocol followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The study was approved by the Ethical Committee of Animal Use and Protection at Ningbo University Health Science Centre (Ethical approval number: NBU20220112). The handling of animals also followed the consensus author guidelines on animal ethics.

**Western blot**

Gastrocnemius muscles were homogenized using an ultrasonic breaker (Fluke, Shanghai, China) in a lysis buffer PMSF dissolution buffer with a concentration of 1mM: RIPA, 1 PMSF, and protease inhibitors (Beijing Solarbio Science&Technology Co., Ltd). Proteins were separated using SDS-PAGE (10% acrylamide) and transferred to PVDF membranes. The primary antibodies and their dilutions were as follows: Beta-actin (1:3000; 20536-1-AP), IDH2 (1:1500; 15932-1-AP), NRF2 (1:1000; 16396-1-AP), AMPKα2 (1:1000; 18167-1-AP), ATP50 (1:1500; 16396-1-AP), SIRT3 (1:1000; 10099-1-AP), SOD2 (1:8000; 10099-1-AP), SIRT1 (1:800; 13161-1-AP), PGC-1α

(1:5000; 66369-1-IP), FOXO3a (1:2000; 10849-1-AP), anti-mouse IgG (H+L) (1:3000; SA00001-1), and anti-rabbit IgG (H+L) (1:3000; SA00001-2). All antibodies were purchased from Proteintech Co. Ltd (Wuhan, China). The protein bands in the blots were visualized using a WESTAR Supernova detection kit (Cyanagen, Bologna, Italy) and ChemiDocTM MP System (BioRad, USA). Band intensity was determined with ImageJ densitometry analysis.

**Analysis of mRNA transcription**

Total RNA was extracted from the gastrocnemius muscle using a *TransZol* Up Kit (Cat. No. ET111-01, Tansgen Biotech, Beijing, China). The purity of the total RNA was evaluated by NanoDrop One (ThermoFisherScientific, Waltham, USA), and the OD260/OD280 ratio of the samples within the range (1.8 ~ 2.1) was used for further analysis. The integrity of total RNA was assessed by denatured gel electrophoresis. Qualified samples should conform to the standard so that the clear bands of rRNA (23S, 18S, and 5S) appear, and the 28S rRNA band should be approximately twice as bright as the 18S rRNA band. Two micrograms of each total RNA sample aliquot were 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 the *EasyScript*® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (One-Step gDNA Removal) Kit (Cat. No. AE341-02, Tansgen Biotech, Beijing, China). Quantitative PCR was performed using the *PerfectStart*® Green qPCR SuperMix Kit (Cat. No. AQ601-02, Tansgen Biotech, Beijing, China). The primers were designed according to the RefSeq mRNA of each gene, and the specificity of each pair of primers was selected and confirmed by NCBI Primer-blast against the RefSeq mRNA database of Mus musculus (Taxid:10088). All pairs of primers were designed to span the intron-exon region except the primers for Nrf2 and β-actin. Forward and reverse primers for the corresponding gene expression are listed in Table 1. For evaluating PCR efficiency, ten-fold serial dilutions

of target gene plasmid cDNA were used to create standard curves for each gene. The amplification efficiencies of all primers were within the 95% ~ 101% range.

The PCR reaction system, including 0.5μL of cDNA, 0.4μM of forward and reverse primers, and 10μL of 2×*PerfetStart*® Green qPCR SuperMix, was finally adjusted to the required total volume of 20 μL 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, 57°C for 15s, and 72 °C for 10 s. The melting curves were tested following amplification by slowly heating from 60 °C to 95 °C in increments of 0.5 °C/s. We used continuous fluorescence collection to confirm that the peak signal was produced only from the target genes during this process. Relative quantification methods (2ˆ-Delta Ct) were used to calculate the relative transcriptional level of each gene according to the cycle threshold value (Ct), in which the expression of genes was normalized against β-actin.

**Malondialdehyde (MDA) analysis**

Malondialdehyde (MDA) concentrations in plasma or muscle tissue were obtained using a Microscale malondialdehyde assay kit (Cat. No. A003-2, Nanjing Jiancheng Biotech Company). The assay kit can detect MDA ranging from 0 to 113nmol/ml, and the intra-assay variation was < 2%. All assays were performed and completed following the manufacturer's instructions.

**Statistical analysis**

Statistical analysis was performed using standard SPSS software (IBM, version 22, Armonk, NY, USA). The values obtained from biological samples were analyzed using two-way or one-way ANOVA (*post hoc* Tukey test). Results were expressed as Box-and-Whisker plots, which include mean, median, first and third quartile, and maximum and minimum for the eight mice in each group ($n$ = 8), and values of $p < 0.05$ were considered statistically significant.

## Results

### Nrf2 expression and associated downstream genes

Fig. 1a and Fig. 1b show the Nrf2 (protein and mRNA) levels in the gastrocnemius muscle of mice. In the overall comparison, the administration of ASX significantly enhances Nrf2 (protein and mRNA) levels, as observed by comparing the two major groups (ASX vs. non-ASX; $F_A$ = 11.123, $p$ = 0.002, and $F_A$ = 141.41, $p$ < 0.001, respectively). There is a weak interaction effect on Nrf2 protein levels ($F_{A\times B}$ = 2.783, $p$ = 0.106) but a strong interaction effect on Nrf2 mRNA ($F_{A\times B}$ =164.41, $p$ < 0.001) between the treatment of ASX and the intervention of HIIT. Multiple comparisons were followed by *post hoc* Tukey tests. Compared with the SC and EC groups, Nrf2 protein quantity in the EA group increased ($p$ = 0.003 and $p$ = 0.004, respectively). There is a higher level of Nrf2 protein in the EA group than in the SA group, but the finding does not reach a significant point ($p$ = 0.098). Compared to all other groups, mRNA *Nrf2* expression in the EA group increased substantially ($p$ < 0.001). In addition, the transcription of *Nrf2* in the EC group was inhibited and markedly lower than in the SC group ($p$ < 0.001).

We subsequently detected the transcript level of *NAD(P)H quinone oxidoreductase* 1 (*NQO1*) and *glutamylcysteine ligase catalytic subunit* (*GCLC*), which are the downstream effectors of Nrf2 (Fig. 1c and 1d). The two-way ANOVA results show that administration of ASX affected the mRNA level of NQO1 and GCLC when comparing the two groups (ASX vs. non-ASX; $F_A$ = 23.06, $p$ < *0.001* and $F_A$ = 79.88, $p$ < 0.001, respectively). The significant effects on *NQO1* and *GCLC* mRNA by HIIT were observed by comparing groups (active vs. inactive; $F_B$ = 43.01, $p$ < 0.001 and $F_B$ = 76.91, $p$ < 0.001, respectively). Significant interaction effects on *NQO1* and *GCLC* were observed between the ASX supplement and the HIIT intervention ($F_{A\times B}$ = 122.66, $p$ < 0.001 and $F_{A\times B}$ = 43.09, $p$ < 0.001, respectively). The multiple comparisons were followed using the *post hoc* Tukey test. *NQO1* and *GCLC* mRNA in the EA group

increased compared to all other groups ($p < 0.001$). Transcriptional *NQO1* decreased in the SA and EC group compared with the SC group ($p < 0.001$ and $p = 0.02$, respectively).

**AMPK, SIRT1 and SIRT3 and MDA concentration**

AMPK, SIRT1, and SIRT3 are upstream modulators involved in redox balance and mitochondrial biogenesis. Fig. 2 shows AMPK and SIRT1/SIRT3 and MDA content between groups in mice muscle. In the overall comparison, administration of ASX enhances AMPK protein levels, as observed by the comparison of groups (ASX vs. non-ASX; $F_A = 7.77$, $p = 0.009$). There is a significant interaction effect on AMPK protein quantity between the administration of ASX and the intervention of HIIT ($F_{A\times B} = 17.08$, $p < 0.001$). Results of *the post hoc* Tukey test suggested that the AMPK protein level in mice gastrocnemius increased in the EA group compared to the EC group ($p < 0.001$; Fig. 2a). AMPK protein quantity in the EC group was lower than in the SC group ($p = 0.015$; Fig. 2a).

The overall comparisons showed that treating ASX promotes SIRT1 and SIRT3 protein quantity by comparing ASX and non-ASX groups ($F_A = 79.08$, $p < 0.001$, and $F_A = 130.61$, $p < 0.001$, respectively). It was demonstrated that the HIIT intervention significantly affects SIRT1 and SIRT3 protein levels when groups were compared (active vs. inactive, $F_B = 17.92$, $p < 0.001$, and $F_B = 83.46$, $p < 0.001$, respectively). There is a significant interaction effect on SIRT1 protein level between the ASX supplementation and the HIIT intervention ($F_{A\times B} = 16.61$, $p < 0.001$). In the *post hoc* Tukey test, SIRT1 protein quantity increased in the EA group compared to the EC group ($p < 0.001$; Fig. 2b). SIRT1 protein level was lower in the EC group than that observed in the SC group ($p < 0.001$; Fig. 2b). The SIRT1 protein quantity in both the EA and SA groups were higher than in the SC group ($p = 0.013$ and $p = 0.010$, respectively). The protein level of SIRT3 increased in the EA group, compared to all other groups ($p < 0.001$; Fig. 2c), and the SIRT3 level rose markedly in the SA group, compared with

the SC group ($p < 0.001$; Fig. 2c). SIRT3 level in the EC group was higher than in the SC group ($p < 0.001$; Fig. 2c).

In the overall comparisons, the groups treated with ASX decreased the MDA level compared to those without ASX treatment ($F_A = 203.01$, $p < 0.001$). It was demonstrated that the HIIT intervention increased the MDA level in the active vs. inactive group ($F_B = 701.18$, $p < 0.001$). There was a significant interaction effect on MDA between the ASX supplementation and the HIIT intervention (($F_{A\times B} = 196.95$, $p < 0.001$). The multiple comparisons were followed by the *post hoc* Tukey test. MDA was elevated in the EC group (nearly 8-fold higher) compared to the SC and SA groups and about 3-fold higher than the EA group ($p < 0.001$; Fig. 2d). The MDA in the EA group was higher compared to both inactive SC and SA groups ($p < 0.01$; Fig. 2d).

**FOXO3a, SOD2, and GPx4 in mice muscle**

FOXO3a, a downstream modulator of AMPK and SIRT1, can activate several antioxidant genes, including *SOD2*. In the overall comparisons, there was a significant effect of ASX on FOXO3a protein level, as observed by comparing two of the major groups (ASX vs. non-ASX; $F_A = 19.03$, $p < 0.001$). In addition, there is an interaction effect between ASX and HIIT groups $F_{A\times B} = 29.40$, $p < 0.001$). In the following multiple comparisons, FOXO3a protein quantity decreased in the EC group, compared with all other groups ($p < 0.01$; Fig. 3a). FOXO3a protein level in the EA group was higher than in the SA group ($p = 0.015$; Fig.3a).

The overall comparisons show that supplementation of ASX significantly affected the SOD2 (protein and mRNA) levels when comparing two major groups (ASX vs. non-ASX; $F_A = 83.66$, $p < 0.001$ and $F_A = 134.06$, $p < 0.001$, respectively). Meanwhile, the HIIT intervention substantially affected the SOD2 transcription ($F_B = 8.56$, $p = 0.007$) when comparing the two major groups (active vs. inactive). Additionally, significant interaction effects on the SOD2 protein level were observed between the ASX administration and the HIIT intervention ($F_{A\times B} = 13.82$, $p < 0.001$). The multiple

comparisons were followed using the post hoc Tukey test. SOD2 (protein and mRNA) increased in SA and EA compared to both SC and EC groups ($p < 0.01$; Fig. 3b and 3d). SOD2 protein also seems to be higher in the SA group than in the EA group, but there was non-significance ($p = 0.06$; Fig.3b). However, *SOD2* mRNA was higher in the SA group than in the EA group ($p = 0.02$; Fig. 3d).

In the overall comparisons, it was suggested that supplementation of ASX significantly affects the transcript level of *Gpx4*, as observed between the two major groups (ASX vs. non-ASX; $F_A = 1035.37$, $p < 0.001$). The HIIT intervention significantly affects *GPx4* transcription, as observed by comparing the two major groups (active vs. inactive; $F_B = 48.97$, $p < 0.001$). Meanwhile, there is a significant interaction effect on *GPx4* transcription between the ASX supplementation and the HIIT intervention ($F_{A\times B} = 101.15$, $p < 0.001$). In the *post hoc* Tukey test, the transcript level of *GPx4* in the EA group was nearly twice that observed in the SC and EC groups ($p < 0.001$; Fig. 3c). *GPx4* in the SA group was higher compared to the EA group ($p < 0.001$) and nearly three-fold greater than the SC group ($p < 0.001$; Fig. 3c).

**PGC-1α in mice muscle.**

To ascertain the effects of ASX on mitochondrial biogenesis, we examined both PGC-1α protein and mRNA levels. We also assayed *NRF1* and *Tfam* transcription, downstream factors regulated by PGC-1α. The overall comparisons between ASX and non-ASX groups exhibit a significant effect on PGC-1α protein and mRNA levels ($F_A = 46.89$, $p < 0.001$ and $F_A = 34.46$, $p < 0.001$, respectively). Additionally, there is a significant interaction effect on the PGC-1α protein levels between the ASX administration and the HIIT intervention ($F_{A\times B} = 26.82$, $p < 0.001$ and $F_{A\times B} = 107.70$, $p < 0.001$, respectively). In the *post hoc* Tukey test, PGC-1α protein quantity was increased in the EA group compared to the EC group ($p < 0.001$; Fig. 4a). Moreover, PGC-1α protein level was higher in EA compared to the SC and SA groups ($p < 0.001$; Fig. 4a). PGC-1α protein level is slightly lower in the EC group than in the SC group,

but non-significance was observed ($p = 0.07$; Fig. 4a). Meanwhile, the transcript level of *PGC-1α* in the EA group was significantly higher compared to all other groups ($p < 0.01$; Fig. 4b). *PGC-1α* mRNA was lower in the EC group than in the SC group ($p < 0.001$; Fig. 4b). In addition, *PGC-1α* mRNA was lower in the SA group compared to the SC group ($p = 0.02$; Fig. 4b).

NRF1 is a regulator of mitochondrial biogenesis and is transcriptionally activated by PGC-1α. Meanwhile, there was an effect of ASX on *NRF1* transcription when comparing the two major groups (ASX vs. non-ASX; $F_A = 13.31$, $p = 0.001$). Meanwhile, the HIIT intervention significantly affected the transcript of *NRF1* when comparing two major groups (active vs. inactive; $F_B = 15.13$, $p = 0.001$). In addition, a significant interaction effect was observed between the ASX administration and the HIIT intervention ($F_{A\times B} = 78.25$, $p < 0.001$). Fig. 4c displays an increase in *NRF1* transcription in the EA group compared to the EC and SA groups ($p < 0.001$). *NRF1* mRNA was lower in the EC group than in the SC group ($p = 0.008$). NRF1 mRNA was lower in the SA group compared to the SC group ($p = 0.005$). *Tfam* is a downstream regulator of PGC-1α and NRF1, and the two-way AVONA results suggest that a significant effect of ASX on *Tfam* transcription was observed by comparing the two major groups (ASX vs. non-ASX; $F_A = 112.45$, $p < 0.001$). Additionally, the HIIT intervention significantly affected *Tfam* transcription when comparing the two major groups (active vs. inactive; $F_B = 46.81$, $p < 0.001$). Meanwhile, it was observed that there is a significant interaction effect between the ASX treatment and the HIIT intervention ($F_{A\times B} = 39.33$, $p < 0.001$). The *post hoc* Tukey test results show an increase in *Tfam* mRNA in the EA group compared to all other groups ($p < 0.001$; Fig. 4d).

**IDH2 and ATP50 protein in mice muscle.**

Isocitrate dehydrogenase 2 (IDH2) and ATP synthase $H^+$ transporting mitochondrial F1 complex O subunit (APT50) are essential components in mitochondrial metabolism. The overall comparison (ASX vs. non-ASX) showed there were significant effects of

ASX on IDH2 and ATP50 protein quantity ($F_A$ = 22.76, $p$ < 0.001 and $F_A$ = 34.88, $p$ < 0.001, respectively). The comparison (active vs. inactive) exhibited that the HIIT intervention significantly affected ATP50 protein quantity ($F_B$ = 17.83, $p$ < 0.001). There was a significant interaction effect on IDH2 protein level between the ASX supplementation and the HIIT intervention ($F_{A\times B}$ = 7.65, $p$ = 0.01). The results of the *post hoc* test suggested that IDH2 protein quantity increased in the EA group, compared with the EC and SC groups ($p$ < 0.001 and $p$ = 0.002, respectively; Fig. 5a). In addition, the IDH2 protein level in the EC group was lower compared to the SA group ($p$ = 0.048). The IDH2 protein quantity in the EA group seems higher than in the SA group, but no significance was observed ($p$ = 0.069; Fig. 5a). Meanwhile, the ATP50 protein level increased in the EA group compared to all other groups ($p$ < 0.01; Fig. 5b). Additionally, the protein level of ATP50 increased in SA in comparison to the SC group ($p$ = 0.005).

**Mfn1, Mfn2, and OPA1 expression in mice muscle**

Mitofusin1 (Mfn1), mitofusin2 (Mfn2), and protein optic atrophy 1(OPA1) are factors that regulate mitochondrial fusion in muscle. The overall comparison (ASX vs. non-ASX) showed a significant effect of ASX on the mRNA level of *Mfn1*, *Mfn2*, *and OPA1* ($F_A$ = 45.88, $p$ < 0.001, $F_A$ = 64.49, $p$ < 0.001, and $F_A$ = 27.78, $p$ < 0.001, respectively). The comparison (active vs. inactive) exhibited that the HIIT intervention significantly affected the mRNA level of *Mfn2* and *OPA1* ($F_B$ = 13.92, $p$ < 0.001, and $F_B$ = 63.13, $p$ < 0.001, respectively). In addition, there were significant interaction effects on the mRNA level of *Mfn1*, *Mfn2*, and *OPA1* between the ASX treatment and the HIIT intervention ($F_{A\times B}$ = 44.76, $p$ < 0.001, $F_{A\times B}$ = 144.86, $p$ < 0.001, and $F_{A\times B}$ = 282.73, $p$ < 0.001, respectively). In the *post hoc* Tukey test, the *Mfn1*, *Mfn2*, and *OPA1* gene expression was higher in the EA group compared to SC, SA, and EC groups (p < 0.001; Fig. 6a, 6b, and 6c), while expression was lower in the EC group compared to the SC group ($p$ < 0.001). The transcript levels of Mfn1 and OPA1 were lower in the SA group than in the SC group ($p$ = 0.04 and $p$ < 0.001, respectively; Fig. 6b and 6c).

## Discussion

Exercise training promotes mitochondrial biogenesis, in part, by oxidative eustress. $H_2O_2$ is a primary oxidative stimulant causing physiological adaptation, and the optimum $H_2O_2$ level is estimated to be in the range of 10 to 100 nM [3, 28]. If the concentration of $H_2O_2$ does not reach appropriate levels, an oxidative response for adaptational reactions may be compromised. On the contrary, in situations where values recorded are greater than 100 nM, $H_2O_2$ may cause a state of oxidative distress. High-intensity interval training (HIIT) is a typical training method that promotes mitochondrial biogenesis and function via an increase of the PGC-1α expression [29]. However, if the intensity or duration of exercise exceeds physiological upper limits, particularly in animals, this can cause oxidative damage. The damage may compromise DNA, lipid, and protein, leading to oxidation, which may impair antioxidant capacity, reduce mitochondrial biosynthesis, and degrade muscle fibers [3, 30].

Astaxanthin has previously been shown to be a potent antioxidant in several pathological states [32, 33]. It can reduce exercise-induced oxidative stress and protect cells from oxidative damage by enhancing the resistance of the cell membrane [18]. Studies have also observed that ASX promotes mitochondrial metabolism, inhibits apoptosis, and can reduce mitochondrial dysfunction [31]. Our previous study evaluated the doses of ASX (5, 15, and 30 mg/kg BW) and the quantities of ASX with 15 and 30 mg/kg BW, and they exhibited similar efficacy in antioxidant capacity [7]. Thus, we estimated that the optimized amount of ASX for C57/6L mice might be within the 5 ~ 15 mg/kg BW range. Based on other studies [25–27], we finally chose the ASX dose of 10 mg/kg for the present work. According to the FDA's guidance for clinical use of equivalent conversion of drug or food between mice and humans [32], we estimated that the dose of 10 mg/kg for mice is equal to that of 0.813 mg/kg for humans (e.g., 56.9 mg/day for a 70 kg human). Although there are still no appropriate recommended dosages for humans, no adverse effects have been reported.

Pharmacokinetic data are available from studies that have issued acute doses of 40 and 100 mg, respectively [33–35]. In addition, ASX uptake can be significantly enhanced if ingested with lipid-based formulations [33]. Thus, we used olive oil as a vehicle for the efficient uptake of ASX for the mice used in this study.

Our previous work showed that high-dose ASX supplementation in mice during moderate-intensity swimming training inhibits the expression of several endogenous antioxidant transcriptional factors or enzymes *(Nrf2, NQO-1, GCLC, GCLM*, and *HMOX-1)* in gastrocnemius or myocardial muscle, indicating that ASX compromised exercise-induced oxidative eustress [7]. MDA is a by-product of lipid peroxidation and is often used to detect oxidative stress following exercise [36]. The current study demonstrates that long-term HIIT causes oxidative distress in mice, but ASX supplementation inhibits oxidative damage (as determined by MDA concentration) during long-term HIIT (Fig. 2d).

We observed that supplementation of ASX increased antioxidant- or mitochondria-associated factors and enzymes. In the overall comparison (ASX vs. non-ASX), the results showed that supplementation of ASX increased the levels of antioxidant factors ($Nrf2^{protein(P)\&mRNA(N)}$, $AMPK^{P}$, $FOXO3a^{P}$, and $SIRT3^{P}$) and enzymes ($NQO1^{N}$, $GCLC^{N}$, $SOD2^{P\&N}$, and $GPx4^{N}$). Meanwhile, the administration of ASX significantly affected the factors associated with mitochondrial biogenesis ($SIRT1^{P}$, $PGC\text{-}1\alpha^{P\&N}$, $NRF1^{N}$, and $Tfam^{N}$) and mitochondrial enzymes ($IDH2^{P}$ and $ATP50^{P}$), promoted the expression of the mitochondrial fusion-associated factors, such as $Mfn1^{N}$, $Mfn2^{N}$, and $OPA1^{N}$. Meanwhile, Interactive effects between the ASX supplementation and the HIIT intervention were superimposed on the increases of most antioxidant- or mitochondria-associated factors and enzymes.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is the central antioxidant regulator for transcription factors possessing antioxidant response element (ARE) and can influence

the transcription of several antioxidant enzymes following an oxidative stress insult [37, 38]. In homeostasis, Nrf2 is anchored by Kelch-like ECH-associated protein 1 (Keap1), which leads to Nrf2-ubiquitylation and degradation. However, keap1 can easily be oxidized by ROS to initiate gene transcription [39]. Animal studies suggest that exercise can promote Nrf2-ARE-mediated pathways in several tissues [38]. In the current study, supplementation of ASX increased the protein and mRNA levels of Nrf2 during long-term HIIT exercise (Fig. 1a and 1b).

The GCLC, alongside NQO1, are critical antioxidant enzymes downstream of Nrf2 [40]. Our results demonstrate that the transcript levels of *GCLC* and *NQO1* increased in the ASX-supplemented HIIT group compared to the HIIT control group, suggesting that chronic HIIT caused an excessive accumulation of RONS (leading to oxidative distress as evidenced by our MDA data) and an impairment in the Nrf2-ARE intermediated antioxidant system. However, supplementation of ASX perhaps neutralized excessive RONS produced in HIIT, thus advocating a return to a state of optimum stimulation, leading to the promotion of Nrf2-ARE activities. However, supplementation of ASX in an inactive status seems not to have such effects.

GPx4 is an essential antioxidant enzyme distributed on the cell membrane and in mitochondria, reducing phospholipid peroxidation [41]. As stated, the transcript level of *GPx4* in the ASX-supplemented HIIT group was more than twice that of the HIIT control group, while *GPx4* in the ASX-supplemented sedentary group was four times higher than the inactive control group; this indicates that ASX promoted *GPx4* expression following HIIT. Moreover, ASX exposure seems to induce *Gpx4* expression, and HIIT partially suppresses ASX-induced Gpx4 expression. Although no precise mechanism exists to explain *GPx4* transcription following ASX supplementation, it is possible, however, that ASX can stimulate *GPx* expression via the cAMP response element. Previous studies indicated that ASX activated the cAMP response element-

binding protein [42–44], while it is currently known that the *Gpx4* gene includes the cAMP response element in the upstream cluster [41].

Exercise increases the consumption of ATP, leading to an increase in the AMP/ADP ratio that activates AMPK [45]. In addition, oxidation of cysteine 299 and 304 sites on AMPKα by ROS (e.g., $H_2O_2$) can directly activate AMPK; alternative mutants in cysteine 299 or 304 resulted in diminished or abrogated AMPK activity [22]. Overexpression of a constitutively active form of AMPK can lead to an increase in the $NAD^+$/NADH ratio and subsequently induce the activation of SIRT1 [46]. Our current study shows that the AMPK protein level diminished in the HIIT group but increased in the ASX-supplemented HIIT group, suggesting that long-term HIIT leads to oxidative distress in mice. However, ASX supplementing promotes a cellular adaptative response aligned to the oxidative eustress model. Meanwhile, the current research shows excessive high-intensity exercise impaired the SIRT1 and FOXO3a protein quantity. However, we observed that ASX supplementation promoted the expression of the SIRT1 and FOXO3a by comparing the groups (EA vs. EC) (Fig. 2b and 3a). Meanwhile, administration of ASX enhances the SIRT1 protein level in the SA group, compared with the SC group (Fig. 2b). However, the FOXO3a protein level seems not to be increased in the SA group, compared with the SC group (Fig. 3a). SIRT1 can activate FOXO3a leading to SOD2 transcription [47]. Our data shows that the SOD2 expression partially accords with the status of SIRT1 and FOXO3 (Fig. 3b and 3d). Although we do not have direct evidence that ASX promotes SOD2 by SIRT1/FOXO3a pathway activation, several other studies have demonstrated that ASX can promote SIRT1 protein in several tissues in animal models [48, 49]. Moreover, previous work shows that ASX can promote FOXO3a protein in electrically stimulated hindlimbs of unloaded mice [50]. In further support, our previous findings suggested that high-dose ASX supplementation can increase SOD activity in moderate-intensity swimming training [7]. We, therefore, postulate that ASX stimulates the expression of SOD2 via the SIRT1/FOXO3a axis, aligned to an elevation in protein SIRT3 level.

On the other hand, SIRT3 can deacetylate and activate SOD2 [51]. We observed that either the HIIT intervention or the ASX supplementation stimulated SIRT3 levels, and the ASX supplementation combined with the HIIT intervention superimposed the SIRT3 protein levels (Fig. 2c).

Mitochondrial biosynthesis is a complex process involving multiple signaling pathways [52]. PGC-1α is a critical factor in the regulation of mitochondrial biogenesis [53]. The previous study shows that PGC-1α deficient mice have impaired exercise-induced mitochondrial biogenesis [54]. Exercise-induced AMPK activation subsequently induces the activation of PGC-1α [55]. In addition, SIRT1 deacetylation further activates PGC-1α [46]. Because PGC-1α positively autoregulates its own promoter, the exercise-induced increase in PGC-1α indicates further amplification of the PGC-1α pathway [56]. Our study shows that PGC-1α protein and mRNA increased in the ASX-supplemented HIIT group compared with the HIIT control group. In contrast, *NRF1* and *Tfam* mRNA increased in the ASX-treated HIIT and sedentary group, compared with the HIIT or inactive control group (Fig. 4c and 4d). Previous work shows that PGC-1α and NRF1 synergistically stimulate the expression of Tfam [53, 57]. Subsequently, Tfam attaches to the promoter region of the mitochondrial DNA (mtDNA), including subunits of four mitochondrial respiratory complexes, NADH dehydrogenase subunits (ND1–6 and ND4L), cytochrome b, cytochrome c oxidase subunits (I–III), and ATP synthase subunits (A6, A8) [58]. In contrast, the mitochondrial biogenesis process is coordinated between mtDNA and nuclear DNA compartments [59]. Incidentally, Tfam overexpression can reduce ROS production in mitochondria, protect mitochondrial DNA from oxidative stress, and significantly improve mitochondrial function [60]. As a result, SIRT1 can promote mitochondrial biogenesis by mediating the PGC-1α/NRF1/Tfam signaling pathway.

In the present study, muscle *Mfn1, Mfn2, and OPA1* transcription following ASX and HIIT were significantly higher compared to HIIT alone and sedentary control mice. It is possible that PGC-1α also stimulates mitochondrial biogenesis by upregulating *Mfn1* and *Mfn2* through ERRα activation [61]. ATP50 and IDH2 are critical enzymes involved in mitochondrial metabolism, while ATP50 and IDH2 can indirectly reflect the number of mitochondria present in tissues. Our study shows that ASX administration in exercise training can increase ATP50 and IDH2, illustrating that ASX promotes mitochondrial proliferation in high-intensity interval training.

In this study, we observed the protein quantity or transcript level of many factors, such as PGC-1α, AMPK, Foxo3a, Sirt1, Sirt3, Nrf2, and NRF1. However, we did not assess the protein-protein interaction or protein-DNA interaction effects (nuclear transcriptional activity) in this study. Thus, this is a limitation of the current study. In the future, the chromatin immunoprecipitation (ChIP) sequencing analysis will be implemented to evaluate Nrf2, NRF1, and PGC-1α transcriptional activity on their downstream genes, while the immunoprecipitation will be used to assess the interaction activity among AMPK-PGC-1α, SIRT1-PGC-1α, and SIRT3-SOD2.

## Conclusion

This novel study shows that astaxanthin protects HIIT mice from oxidative distress by upregulating specific antioxidant pathways and facilitating mitochondrial biogenesis. The use of astaxanthin needs further evaluation concerning oxidative stress and chronic exercise training. However, at this juncture, and based on the findings of this study, supplementation of astaxanthin is beneficial under exercise conditions that promote oxidative distress. It may be unnecessary, however, to supplement with astaxanthin when physiological exertion is within the range of oxidative eustress.

## Supplementary material

The mice feed composition used in this study is listed in the file.

## Acknowledgment

This study was sponsored by the Ningbo Natural Science Foundation (No. 2019A610347).

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## Tables

**Table 1 Primers of each gene used for real-time PCR**

| Name | Accession number | Primers (5'-3') | Amplicon length (bp) |
|---|---|---|---|
| *Nrf2* | NM_010902.5 | F: GCTCCTATGCGTGAATCCCAA<br>R: TTTGCCCTAAGCTCATCTCGT | 143bp |
| *NQO1* | NM_008706.5 | F: CGCCTGAGCCCAGATATTGT<br>R: GCACTCTCTCAAACCAGCCT | 124bp |
| *GCLC* | NM_010295.2 | F: TGCACATCTACCACGCAGTC<br>R: ATCGCCTCCATTCAGTAACAAC | 128bp |
| *GPx4* | NM_001037741 | F: CCAAGGACATCGACGGGCAC<br>R: AAGGCCAGGATTCGTAAACCAC | 163bp |
| *SOD2* | NM_013671.3 | F: CTCAGGTCGCTCTTCAGCC<br>R: CCTTGGACTCCCACAGACAC | 193bp |
| *PGC1α* | NM_008904.2 | F: ATCAAGCCACTACAGACACC<br>R: TTTCAGACTCCCGCTTCTCG | 170bp |
| *NRF1* | NM_001164226.1 | F: AAAGAGACAGCAGACACGTT<br>R: CTCTTGTACTTTCGCACCACA | 171bp |
| *Tfam* | NM_009360.4 | F: AAGGATGATTCGGCTCAGGGA<br>R: TCCGGATCGTTTCACACTTCG | 198bp |
| *Mfn1* | NM_024200.4 | F: AGCCATCACTGCAATCTTCGG<br>R: ACAGCACCTCCCCAATGACA | 167bp |
| *Mfn2* | NM_001285922.1 | F: GGTCAGGGGTATCAGCGAAG<br>R: TGACCAATCCCAGATGGCAG | 132bp |
| *OPA1* | NM_001199177.1 | F: TCTGAGGCCCTTCTCTTGTT<br>R: TTTTCCAGGCGCTCCAAGAT | 179bp |
| *β-actin* | NM_007393.5 | F: GCGCAAGTACTCTGTGTGGA<br>R: CAGCTCAGTAACAGTCCGCC | 146bp |

## Figures

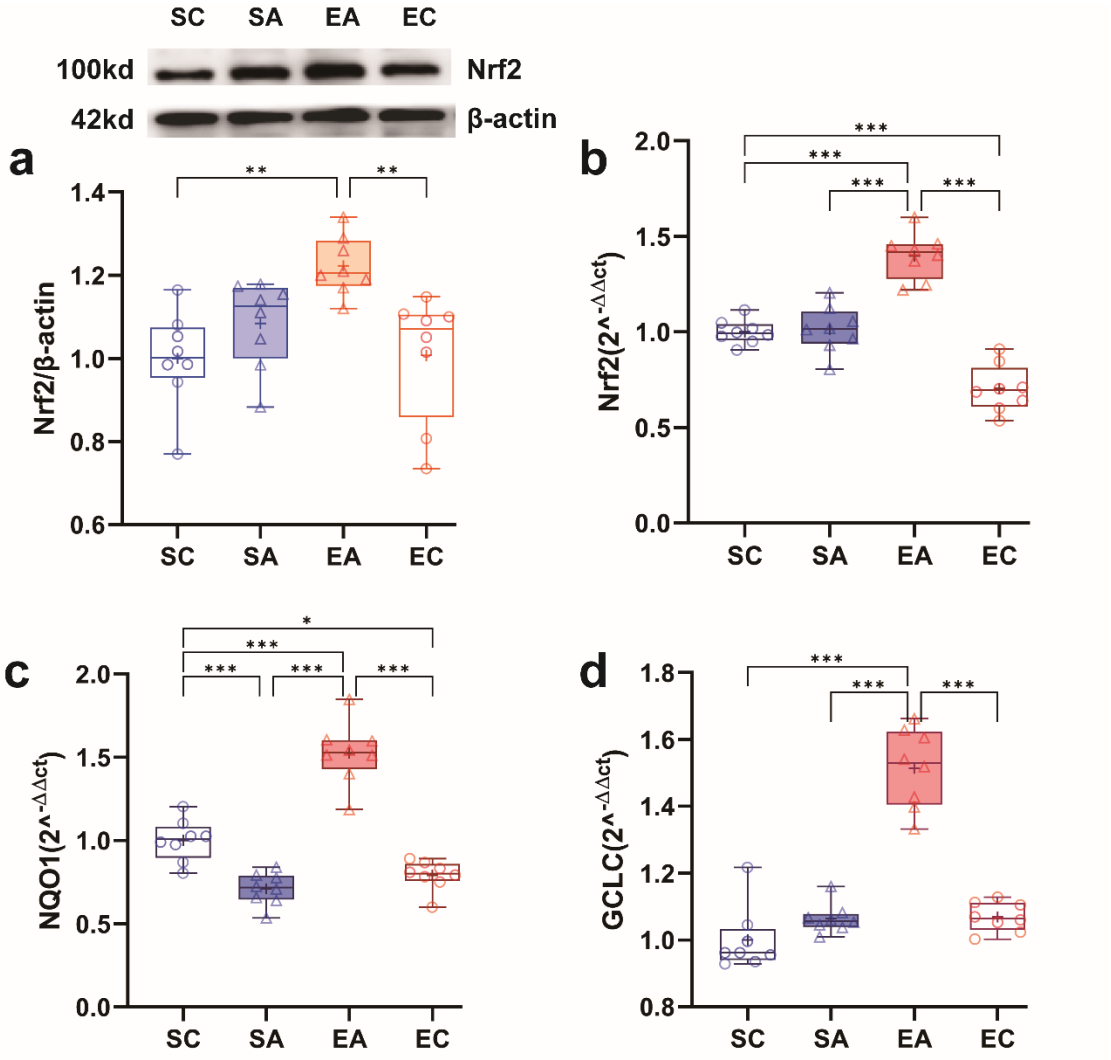


**Fig. 1 Nrf2, NQO1, and GCLC in mice gastrocnemius muscle.** SC, SA, EA, and EC represent each group. **a**: The protein level of Nrf2. **b**: mRNA level of *Nrf2*; **c**: mRNA level of *NQO1*; **d**: mRNA level of *GCLC*. All data are normalized to SC, and values are expressed as Box-and-Whisker plots, 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. *significant difference at $p < 0.05$; **significant difference at $p < 0.01$; ***significant difference at $p < 0.001$

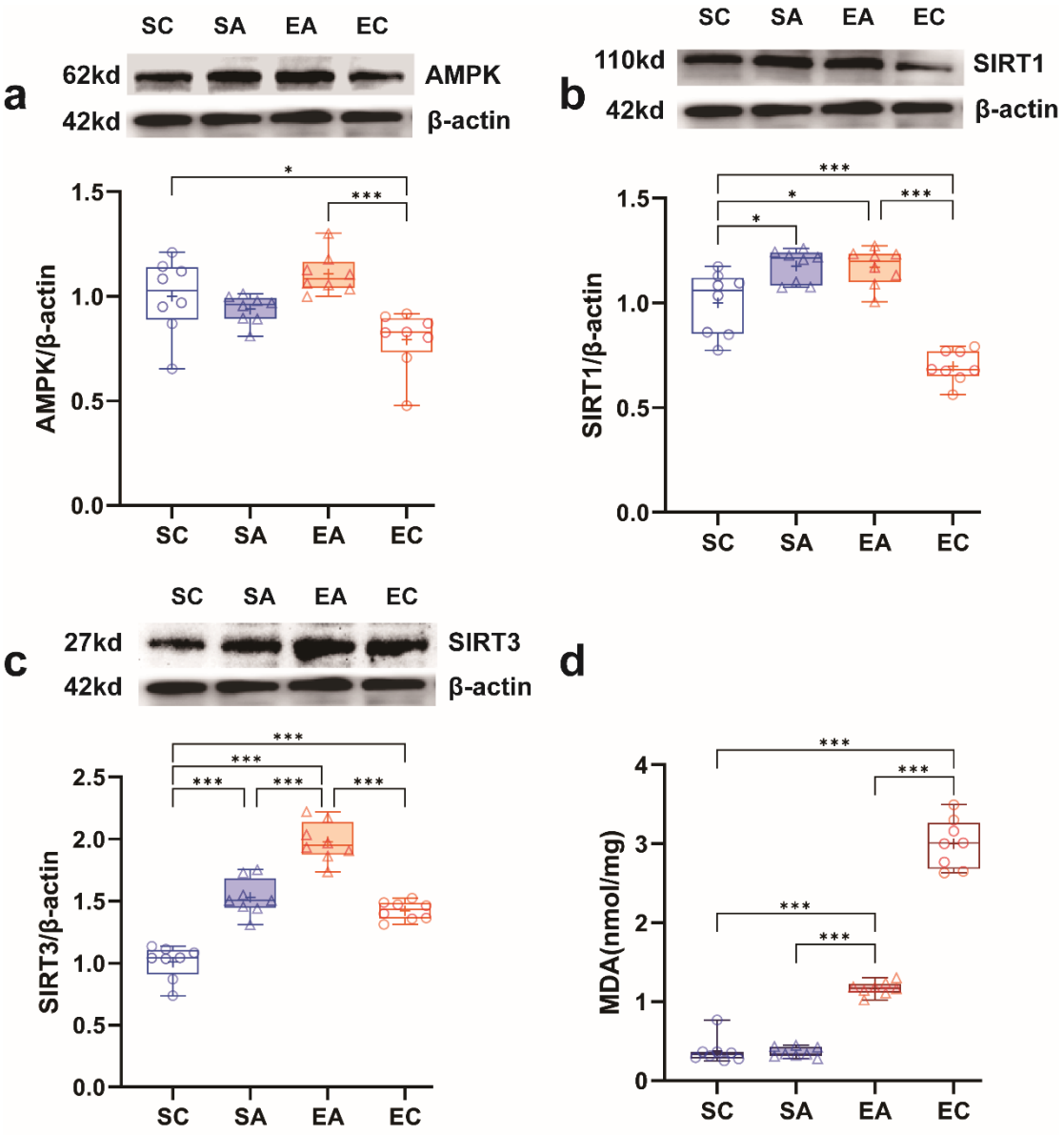


**Fig. 2 AMPK, Sirt1, and Sirt3 and MDA in mice muscle.** SC, SA, EA, and EC represent each group. **a**: AMPK protein; **b**: SIRT1 protein; **c**: SIRT3 protein; **d**: MDA amount. All data are normalized to SC, and values are expressed as Box-and-Whisker plots, in which the bottom and top of the box 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. *significant difference at $p < 0.05$; **significant difference at $p < 0.01$; ***significant difference at $p < 0.001$

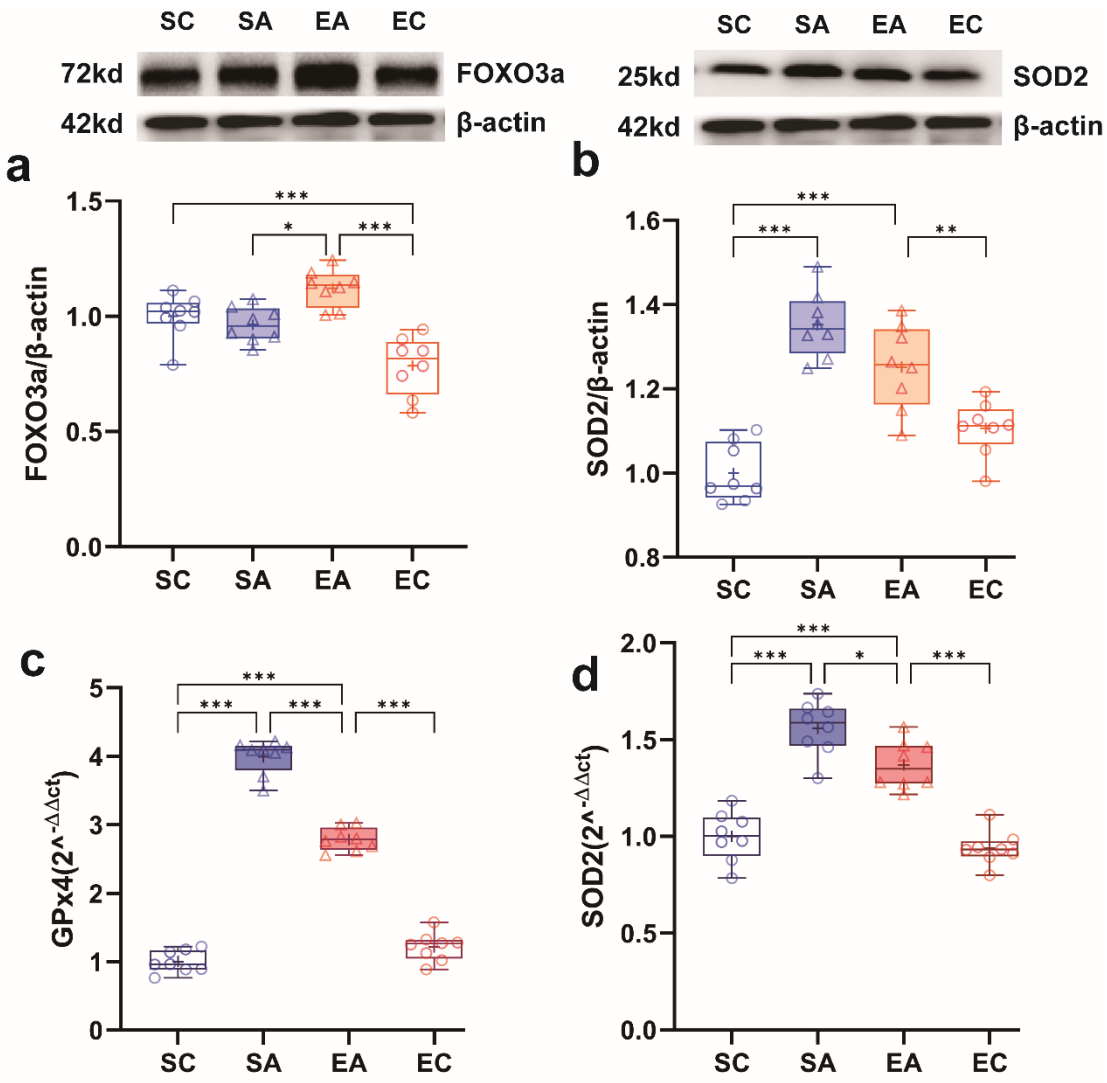


**Fig. 3 FOXO3a, SOD2, and GPx4 expression in mice muscle.** SC, SA, EA, and EC represent each group. **a**: FOXO3a protein; **b**: SOD2 protein; **c**: *GPx4* mRNA; **d**: SOD2 mRNA. All data are normalized to SC, and values are expressed as Box-and-Whisker plots, in which the bottom and top of the 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. *significant difference at $p < 0.05$; **significant difference at $p < 0.01$; ***significant difference at $p < 0.001$

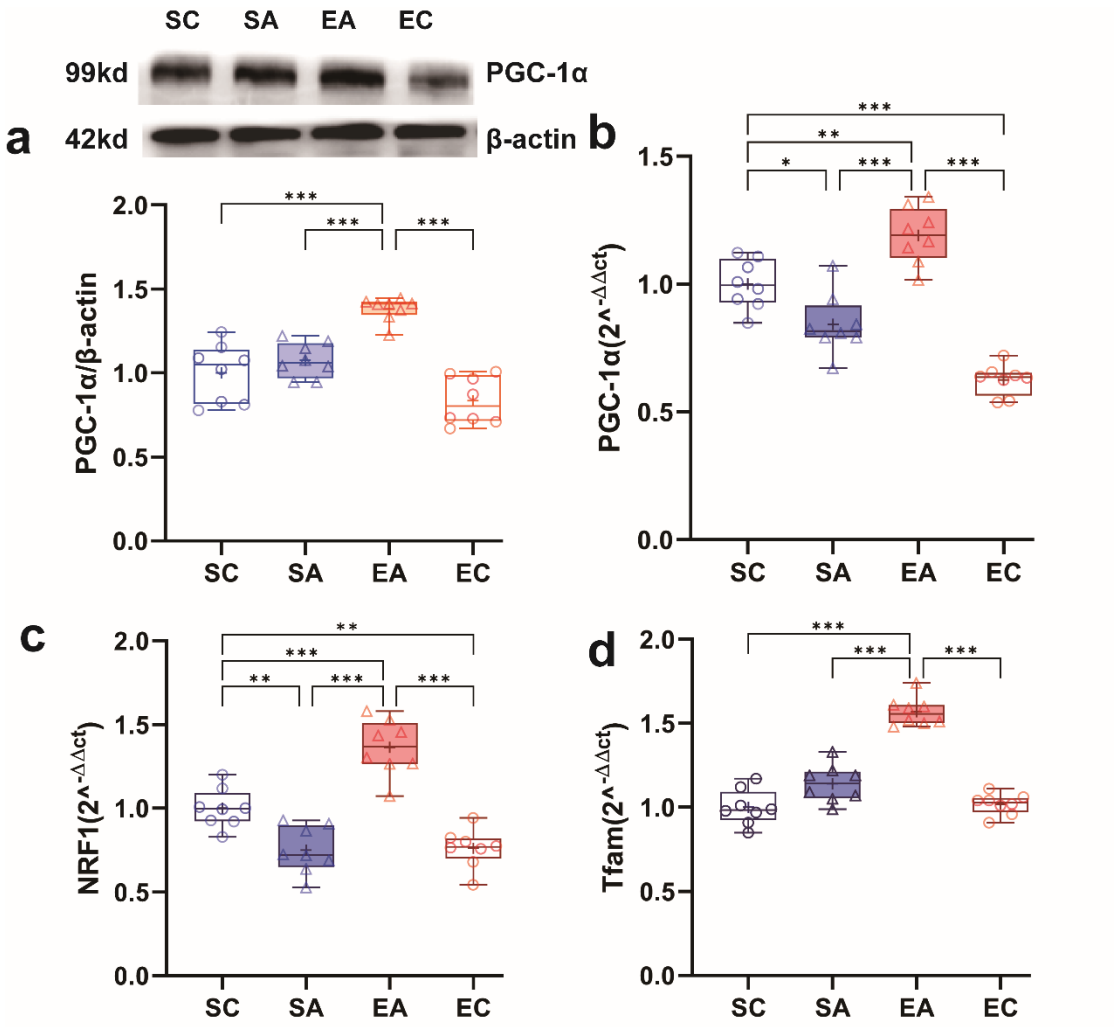


**Fig. 4 PGC-1α, NRF1, and Tfam in mice muscle.** SC, SA, EA, and EC represent each group. **a**: Protein level of PGC-1α; **b**: PGC-1α mRNA; **c**: NRF1 mRNA; **d**: Tfam mRNA. All data are normalized to SC, and values are expressed as Box-and-Whisker plots, in which the bottom and top of the box present 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. *significant difference at $p < 0.05$; **significant difference at $p < 0.01$; ***significant difference at $p < 0.001$

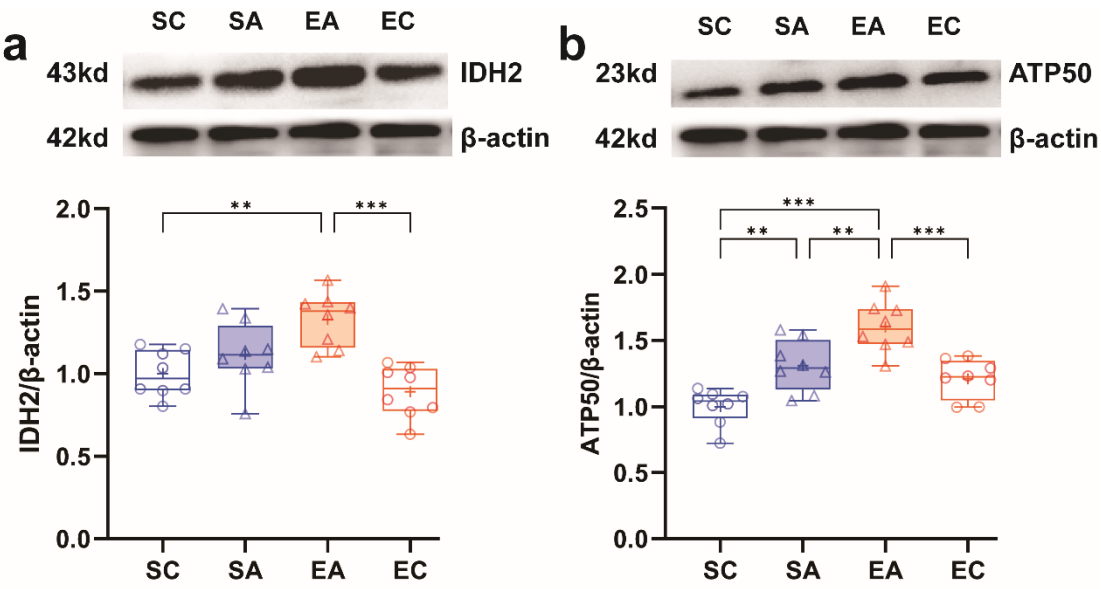


**Fig. 5 IDH2 and ATP50 in mice gastrocnemius muscle.** SC, SA, EA, EC represents each group. **a**: IDH2 protein; **b**: ATP50 protein. All data are normalized to SC, and values are expressed as Box-and-Whisker plots, in which the bottom and top of the box present the first and third quartile, respectively; 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. *significant difference at $p < 0.05$; **significant difference at $p < 0.01$; ***significant difference at $p < 0.001$

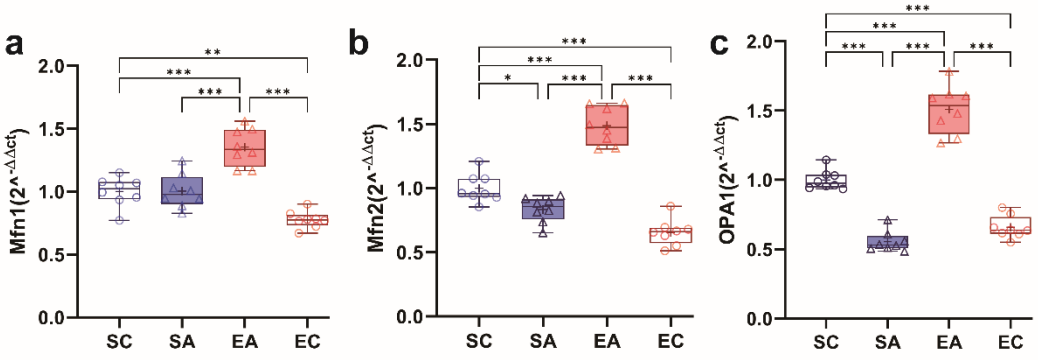


**Fig. 6 *Mfn1*, *Mfn2*, and *OPA1* expression in mice muscle.** SC, SA, EA, and EC represent each group. **a**: *Mfn1* mRNA; **b**: *Mfn2* mRNA; **c**: *OPA1* mRNA; All data are normalized to SC, and values are expressed as Box-and-Whisker plots, in which the bottom and top of the box present the first 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. *significant difference at $p < 0.05$; **significant difference at $p < 0.01$; ***significant difference at $p < 0.001$