Original Research *Highlight article*

Antifatigue effect of naringin on improving antioxidant capacity and mitochondrial function and preventing muscle damage

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Impact Statement

Naringin is a natural flavanone glycoside found mainly in citrus fruits with many pharmacological effects. To explore effects of naringin on antifatigue ability, we performed weight-loaded and non-loading swimming tests *in vivo*. Our results confirmed that dietary naringin supplementation significantly prolonged the swimming time to exhaustion of mice, enhanced the antioxidant capacity and mitochondrial function, and prevented muscle damage. Our findings suggest that naringin may be developed as a functional food or natural drugs having an antifatigue effect.

Abstract

The aim of this study was to explore effects of naringin (Nar) on antifatigue ability; the weight-loaded and non-loading swimming tests were performed. Compared with the control group, dietary supplementation of Nar significantly prolonged the weight-loaded swimming time to exhaustion of mice ($P < 0.01$). Nar significantly reduced the serum lactic acid (LD) level ($P < 0.05$) and lactate dehydrogenase (LDH) activity ($P < 0.001$), while increased the serum non-esterified free fatty acids (NEFA) level ($P < 0.001$). In addition, Nar significantly increased the liver glycogen and muscle glycogen contents ($P < 0.05$) and the phosphoenolpyruvate carboxykinase (*PEPCK*) ($P < 0.01$) and glucokinase (*GCK*) mRNA levels ($P < 0.001$) in liver and gastrocnemius (GAS) muscle. Furthermore, Nar significantly improved the antioxidant capacity, mitochondrial function, and muscle mitochondrial fatty acid β-oxidation ($P < 0.05$), and decreased inflammation and muscle damage–related gene expression ($P < 0.05$). These findings suggested that Nar can improve antifatigue effect by enhancing antioxidant capacity and mitochondrial function and preventing muscle damage.

Keywords: Naringin, antifatigue, antioxidant capacity, mitochondrial function, muscle damage

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Introduction

Fatigue is a complex physiological and biochemical process,[1] which is related to the exhaustion of energy, the accumulation of free radicals, and metabolites. And fatigue caused by long-term exercise can cause tissues damage. In recent years, more and more studies have found that many plant extracts can safely and effectively relieve fatigue.[2,3]

Flavonoid is a type of plant extract with strong antioxidant effect.[4,5] Naringin (Nar, 4′, 5, 7-trihydroxy flavanone 7-rhamnoglucoside) is a natural flavanone glycoside, which found mainly in citrus fruits with many pharmacological actions such as antioxidant, antibacterial, antiapoptotic and anti-inflammatory.[6] There is evidence that dietary supplementation with 0.04% Nar promotes muscle fiber type transition from fast-switch to slow-switch.[7] Moreover, muscle fatigue has been proved to be directly related to muscle fiber type.[8] Muscle fiber is the basic component of skeletal muscle. Slow-twitch fiber contains more mitochondrial density and resistance to fatigue, while fast-twitch fiber contains less mitochondrial content and easy to fatigue.[9] Therefore, it is reasonable to hypothesize that Nar may play an important role in improving antifatigue effect.

To verify the hypothesis, this study employed the weight-loaded swimming test to explore the role of Nar in regulating antifatigue function in mice. To investigate the antifatigue mechanism, the metabolic parameters and antioxidant status in serum, liver and muscle, glycogen storage, mitochondrial function, and the expression levels of antioxidant and antifatigue-related genes were determined.

Materials and methods

Ethics statement

Animal procedures were performed according to the Guidelines for Care and Use of Laboratory Animals of Sichuan Agricultural University and approved by the Animal Care Advisory Committee of Sichuan Agricultural University under permit no. YYS191204.

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Weight-loaded swimming test

Nar (purity 98%) was obtained from Shanxi Huike Botanical Development Co., Ltd. (Xian, China). Thirty healthy three-week-old male BALB/c mice, purchased from Chengdu Dossy Experimental Animals Co., Ltd. (Chengdu, China), were randomly divided into control group (a basal diet) and 0.04% Nar group (a basal diet supplemented with 400 mg Nar/kg of feed) according to initial body mass ($n = 15$), and mice had free access to water and feed. After six weeks, all mice were subjected to the exhaustive swimming test with 5% of body mass burden until exhaustive, and the swimming time was recorded.[10]

Non-loading swimming test and sample collection

Thirty healthy three-week-old male BALB/c mice, purchased from Chengdu Dossy Experimental Animals Co., Ltd., were randomly divided into the control group (a basal diet) and 0.04% Nar group (a basal diet supplemented with 400 mg Nar/kg of feed). After six weeks, the mice were subjected to swim in water at 30 ± 1°C for 90 min. The blood samples were collected immediately after CO_2 asphyxiation and centrifuged (956*g* at 4°C for 15 min) to prepare serum. Then, gastrocnemius (GAS) muscle and liver samples were collected into a 2-mL Eppendorf tube, immediately frozen in liquid nitrogen, and stored at −80°C.

Biochemical analysis

The serum lactic acid (LD) (Cat. No. A019-2-1) and non-esterified free fatty acids (NEFA) levels (Cat. No. A042-1-1), the contents of liver glycogen and muscle glycogen (Cat. No. A043-1-1) and malondialdehyde (MDA) (Cat. No. A003-1-2), the activities of lactate dehydrogenase (LDH) (Cat. No. A020-2-1), total superoxide dismutase (T-SOD) (Cat. No. A001-1-2), glutathione peroxidase (GSH-Px) (Cat. No. A005-1-2) and catalase (CAT) (Cat. No. A007-1-1), and the total protein content (Cat. No. A045-2) were measured using commercial detection kits purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The reactive oxygen species (ROS) level in the GAS muscle and liver tissue was detected by an ELISA kit purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Cat. No. ml009876-1, Shanghai, China).

Adenosine triphosphate content assay

Adenosine triphosphate (ATP) content in the GAS muscle and liver tissues was determined with an ATP assay kit (Cat. No. A095-1-1, Nanjing Jiancheng Bioengineering Institute).

Cytochrome c oxidase and cytochrome c oxidase III levels analysis

The cytochrome c oxidase (COX) activity (Cat. No. ml37420) and COX III level (Cat. No. ml058157) in the GAS muscle and liver were detected according to the instructions provided by commercially available ELISA kits purchased from Enzyme-linked Biotechnology Co., Ltd.

Real-time quantitative polymerase chain reaction

Total RNA was isolated from GAS muscle and liver tissues using TRIzol reagent (TaKaRa, China). RNA was reverse transcribed into complementary DNAs using a PrimeScript® RT reagent Kit with gDNA Eraser (TaKaRa). All primer sequences are listed in Table 1. Real-time quantitative polymerase chain reaction (PCR) was performed using TB green Premix Ex Taq II (Tli RNaseH Plus; TaKaRa) following the manufacturer's instructions. Results were obtained with $2^{-\Delta\Delta Ct}$ method, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA). All data were presented as the mean values ± standard deviation (SD). Comparisons between two groups were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

Effect of Nar on the loaded swimming time of mice

Compared with the control group, dietary supplementation of 0.04% Nar significantly prolonged the exhaustive swimming time ($P < 0.01$) (Figure 1). On average, the loaded swimming time in 0.04% group increased by 30.51%.

Effect of Nar on biochemical indexes

Compared with the control group, 0.04% Nar treatment significantly increased the NEFA level ($P < 0.001$) and reduced the LDH activity ($P < 0.001$) and LD level ($P < 0.05$) in serum (Figure 2). Furthermore, 0.04% Nar significantly increased the muscle glycogen and liver glycogen contents ($P < 0.05$) and the mRNA levels of phosphoenolpyruvate carboxykinase (*PEPCK*) ($P < 0.01$) and glucokinase (*GCK*) ($P < 0.001$) (Figure 3).

Effect of Nar on antioxidant capacity

Compared with the control group, 0.04% Nar treatment significantly increased the T-SOD, CAT, and GSH-Px activities, and reduced the content of MDA and level of ROS in serum ($P < 0.01$) (Figure 4), and GAS muscle and liver of mice ($P < 0.05$) (Figure 5(A) to (E)). In addition, 0.04% Nar significantly increased the *SOD1*, *SOD2*, *GPX*, *CAT*, and *Nrf2* mRNA levels and decreased the mRNA level of *Keap1* ($P < 0.01$) (Figure 5(F) and (G)).

Effect of Nar on mitochondrial function

Compared with the control group, 0.04% Nar treatment significantly increased the ATP ($P < 0.01$) and COX III contents

Table 1. List of genes, primer sequences, GenBank accession numbers, and product sizes in this study.

Gene name	Primer	Sequence (5'-3')	GenBank accession number	Product size (bp)
PEPCK	Forward	TTGAACTGACAGACTCGCCC	NM_011044	105
	Reverse	GGCACTTGATGAACTCCCCA		
GCK	Forward	TGATGAGCCGGATGCAGAAG	NM_010292	159
	Reverse	CCCTGAAGTTGGTTCCTCCC		
Syncytin1	Forward	ACCAGTAGCTCCCCTTACCA	NM_014590	141
	Reverse	AGAGTGATAGCAGTTGCGGG		
ASCT1	Forward	GTGGTTGCCGCATTCACTAC	AF246129	143
	Reverse	AGGGAAAAAGGACGAGACC		
iNOS	Forward	TCCTGGACATTACGACCCCT	U43428.1	93
	Reverse	AGGCCTCCAATCTCTGCCTA		
TNF-α	Forward	GGATGAGAAGTTCCCAAATGG	AY423855	119
	Reverse	GGTGGTTTGCTACGACGTG		
PPAR-δ	Forward	CCGACATTCCATGTTGAGGC	NM_011145	138
	Reverse	AATGCATGAGCGAGAGGTGG		
CPT1	Forward	ACTGAGACTGTGCGTTCCTG	NM_009948	117
	Reverse	GTGCTTTTCGGAGGCTTTCC		
HADH	Forward	AGGGGTCCGCATTCCATTTC	NM_145558	82
	Reverse	CGAAAGTGCAGCTCTAGCCA		
UCP3	Forward	TGGGAGCTTGGAACGTGATG	NM_009464	159
	Reverse	AGATTCCCGCAGTACCTGGA		
SOD1	Forward	GGAACCATCCACTTCGAGCA	NM_011434	87
	Reverse	CCCATGCTGGCCTTCAGTTA		
SOD2	Forward	CGTGAACAACCTCAACGCC	NM_013671	199
	Reverse	GTCACGCTTGATAGCCTCCA		
CAT	Forward	GCGGATTCCTGAGAGAGTGG	L25069	143
	Reverse	TGGAGAACCGAACGGCAATA		
GPX	Forward	CTCAAGTACGTCCGACCTGG	BC086649	94
	Reverse	TAAAGAGCGGGTGAGCCTTC		
Nrf2	Forward	AAAGCACAGCCAGCACATTC	NM_010902	86
	Reverse	TGGGATTCACGCATAGGAGC		
Keap1	Forward	CTCAACCGCTTGCTGTATGC	BC055732	194
	Reverse	TTCAACTGGTCCTGCCCATC		
GAPDH	Forward	AGGGCATCTTGGGCTACAC	NM_008084	211
	Reverse	TGGTCCAGGGTTTCTTACTCC		

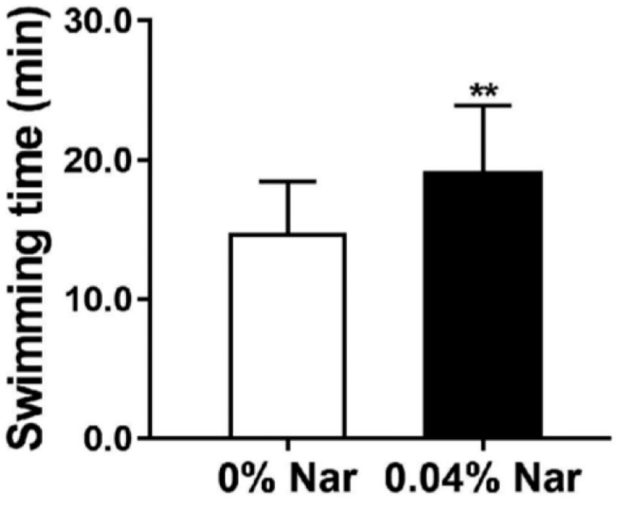

Figure 1. Effect of Nar on weight-loaded swimming time in mice. Data were presented as mean values ± SD.
**$P < 0.01$.

($P < 0.05$) and COX activity ($P < 0.05$) in liver and GAS muscle of mice (Figure 6).

Effect of Nar on the mRNA expression of fatigue-related genes

Compared with the control group, 0.04% Nar significantly increased alanine/serine/cysteine/threonine transporter 1 (*ASCT1*) ($P < 0.05$), carnitine palmitoyltransferase-1 (*CPT1*) ($P < 0.001$), peroxisome proliferator–activated receptor-delta (*PPAR-δ*) ($P < 0.001$), and uncoupling protein-3 (*UCP3*) ($P < 0.001$) mRNA levels, while reduced the mRNA levels of *Syncytin-1* ($P < 0.01$), tumor necrosis factor (*TNF-α*) ($P < 0.01$), and inducible nitric oxide synthase (*iNOS*) ($P < 0.05$). There was no significant difference in β-hydroxyacyl coenzymes A dehydrogenase (*HADH*) mRNA level between 0.04% Nar treatment group and control group (Figure 7).

Discussion

This study found that dietary Nar supplementation increased antifatigue function of mice, and the effect might be mediated by improving antioxidant capacity and mitochondrial function and preventing muscle damage, which was consistent with the hypothesis of this study.

Swimming time is usually used to evaluate the antifatigue effect.[11,12] The formation of fatigue is related to exhaustion

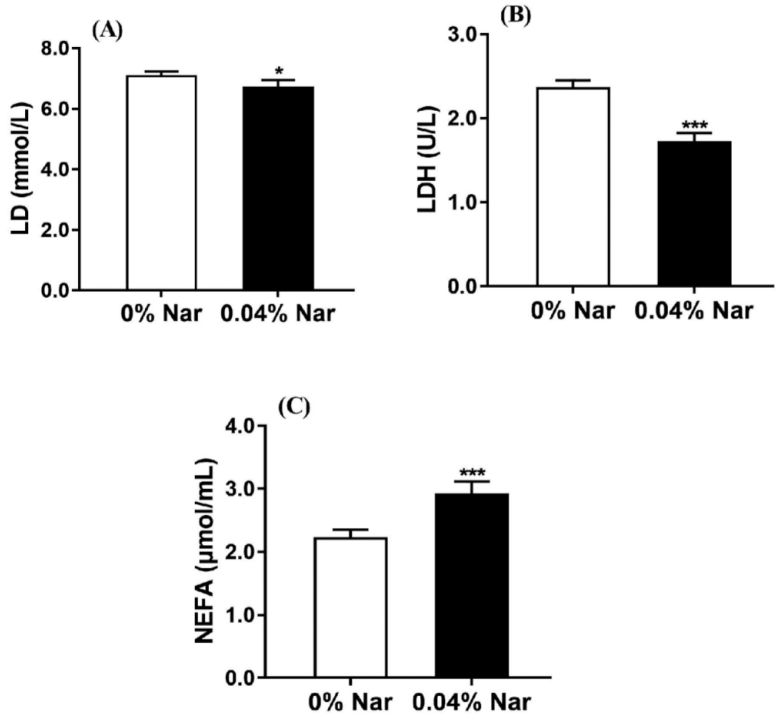

Figure 2. Effect of Nar on LD, LDH, and NEFA in serum: (A) LD level, (B) LDH activity, and (C) NEFA level. Data were presented as mean values ± SD. $^{*}P < 0.05$; $^{***}P < 0.001$.

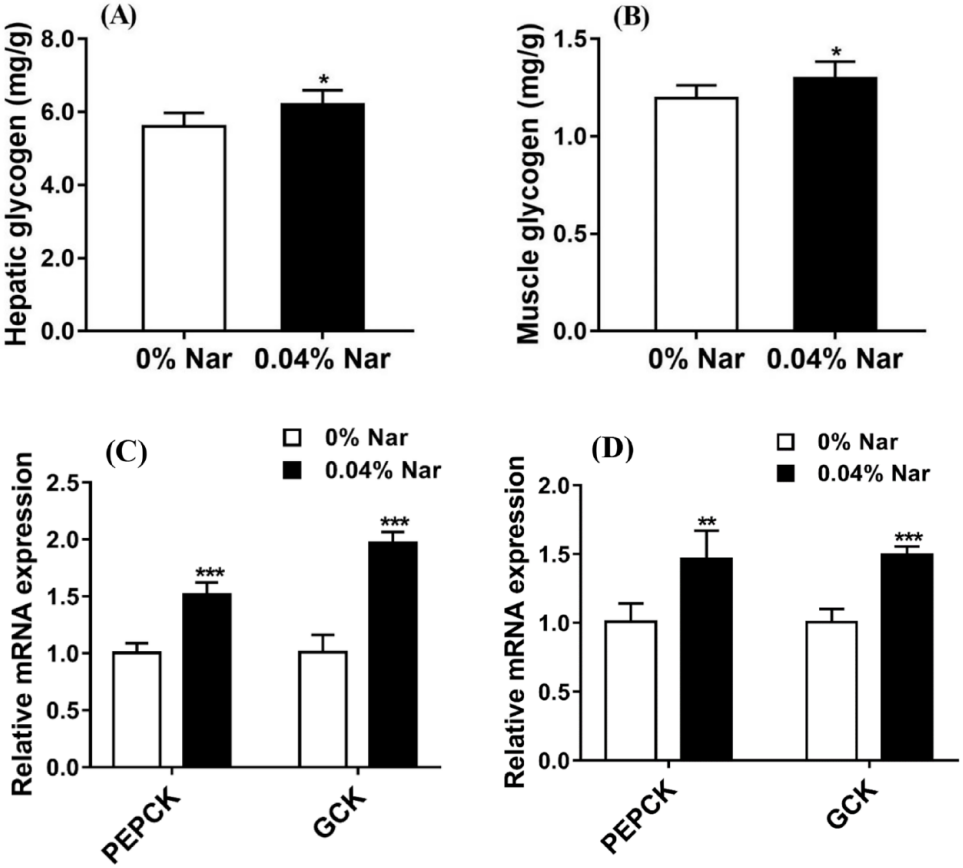

Figure 3. Effect of Nar on glycogen content and the mRNA expression of *PEPCK* and *GCK* in mice: (A) hepatic glycogen content, (B) muscle glycogen content, (C) the mRNA expression of *PEPCK* and *GCK* in liver, and (D) the mRNA expression of *PEPCK* and *GCK* in GAS muscle. Data were presented as mean values ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

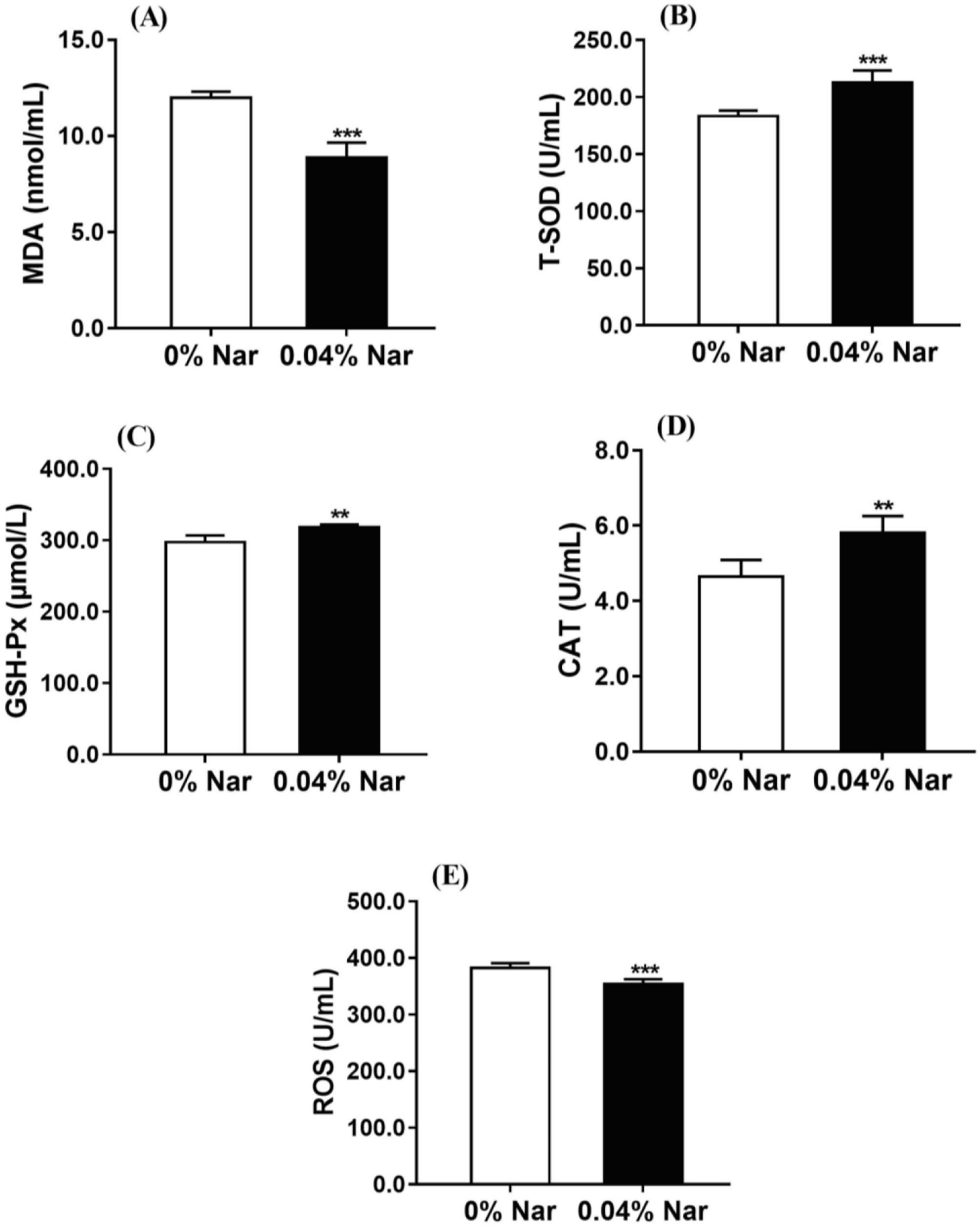

Figure 4. Effect of Nar on serum antioxidant levels: (A) MDA content, (B) T-SOD activity, (C) GSH-Px activity, (D) CAT activity, and (E) ROS level. Data were presented as mean values ± SD.
$^{**}P < 0.01$; $^{***}P < 0.001$.

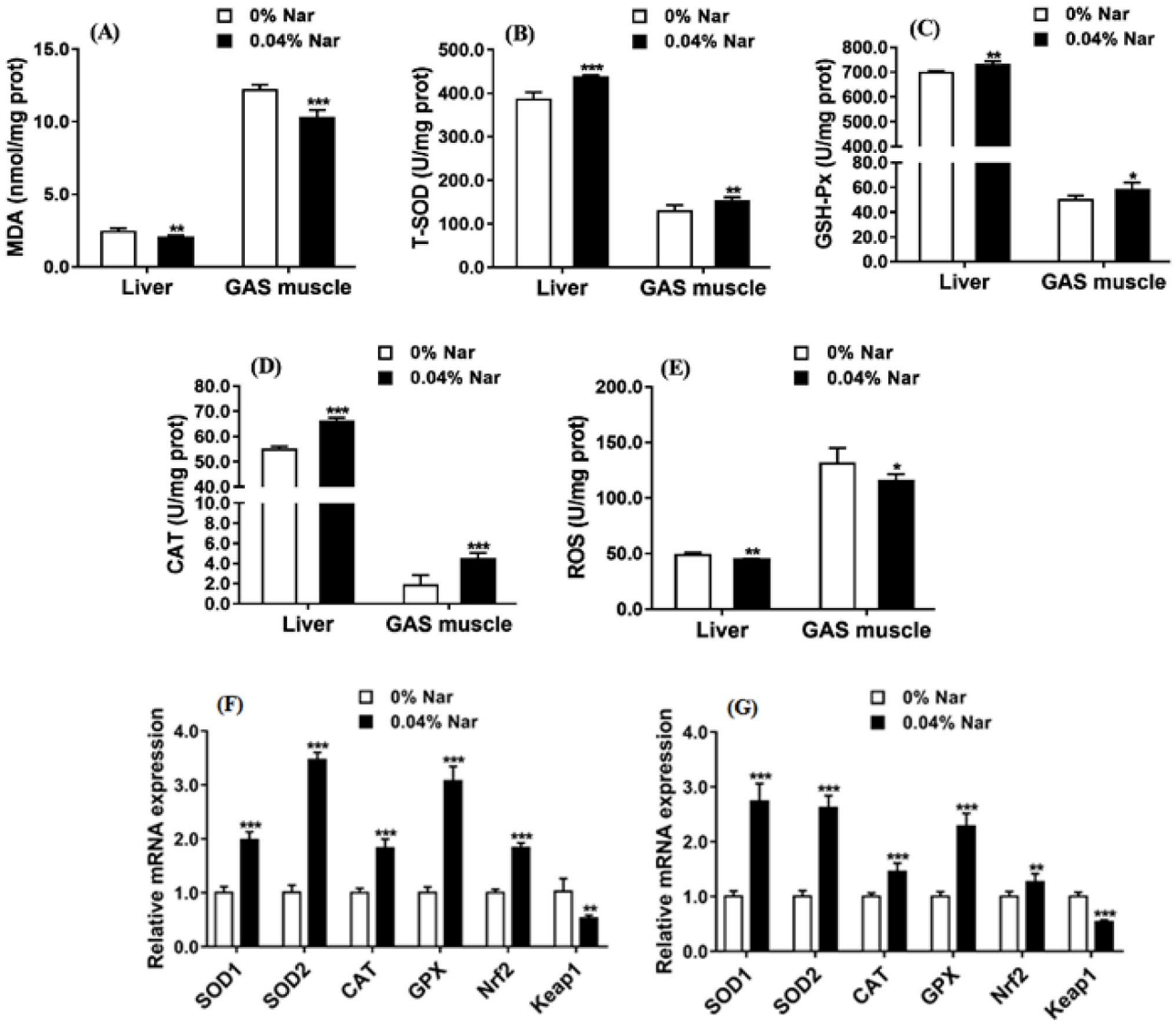

Figure 5. Effect of Nar on liver and GAS muscle antioxidant levels: (A) MDA content, (B) T-SOD activity, (C) GSH-Px activity, (D) CAT activity, (E) ROS level, (F) mRNA expression in liver, and (G) mRNA expression in GAS muscle. Data were presented as mean values ± SD.
$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

of energy materials and the accumulation of metabolites.[13] The LD level has been considered as the most important cause of muscle fatigue.[14] The serum LDH activity reflects the degree of muscle injury.[15] The storage and utilization of energy substances such as glycogen and NEFA are also important indexes to judge the antifatigue effect.[16–18] GCK/PEPCK is an important signaling pathway in regulating energy metabolism, which can maintain glycogen level and improve antifatigue capacity.[19] In this study, dietary Nar supplementation prolonged the exhaustive swimming time and significantly increased the serum NEFA level and liver glycogen and muscle glycogen contents, as well as increased the *GCK* and *PEPCK* mRNA levels in liver and GAS muscle, while reduced the serum LD level and LDH activity. There is evidence that increasing the storage of energy substances such as sugar and fat and reducing the accumulation of metabolites may be the antifatigue effect of plant extracts.[20,21] Taken together, data showed that Nar increased the antifatigue capacity of mice.

It has been found that fatigue is associated closely with the levels of free radicals.[22] ROS levels are considered as one of the important mechanisms of exercise-induced fatigue.[23] Moreover, mitochondria dysfunction results in the increase in the ROS production, and decrease of antioxidant enzyme activities.[24] MDA, an oxidative stress marker, is an end product of lipid peroxidation.[25] T-SOD (including SOD1 and SOD2) is an important antioxidant enzyme, which can specifically remove the oxygen free radicals, and GSH-Px and CAT play an important role in scavenging the hydrogen peroxide.[26] In addition, mitochondria are organelle that produce ATP, and the activity of COX can directly affect the mitochondrial function.[27] COX III is the main constituent subunit of COX, which is responsible for the regulation of COX assembly and antioxidative enzyme activity.[24] In

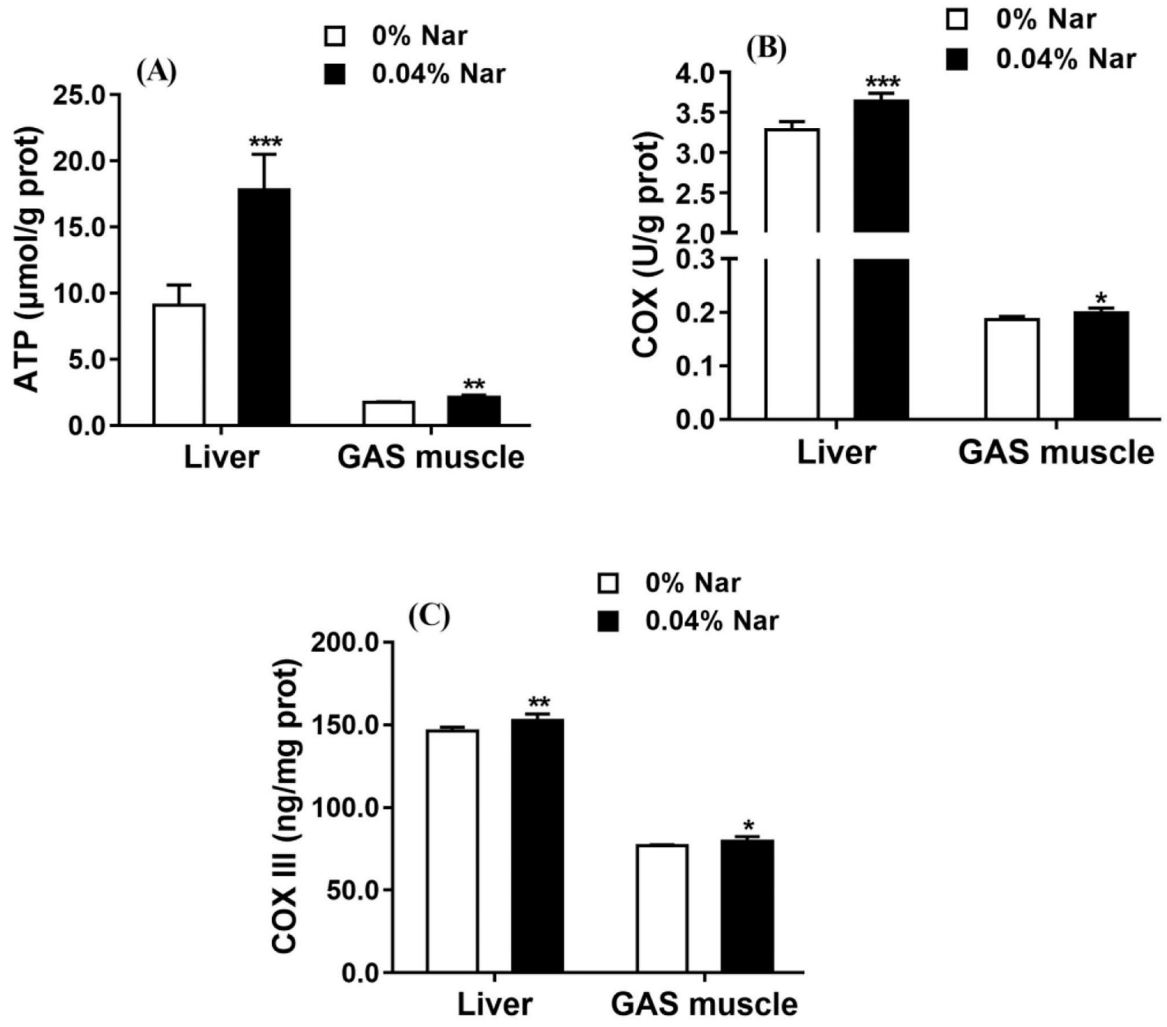

Figure 6. Effect of Nar on mitochondrial function in the liver and GAS muscle of mice: (A) ATP content, (B) COX activity, and (C) COX III content. Data were presented as mean values ± SD.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

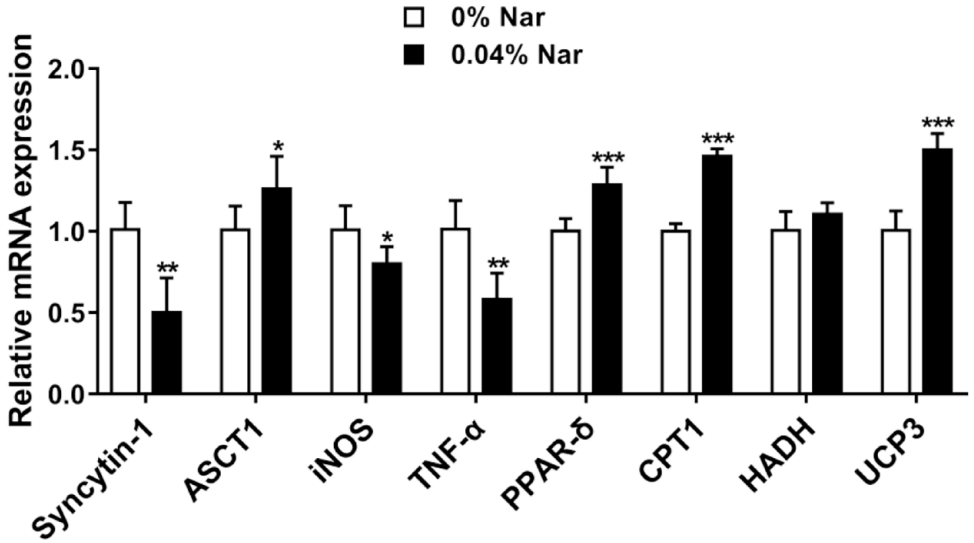

Figure 7. Effect of Nar on fatigue-related gene mRNA expression in GAS muscle of mice. Data were presented as mean values ± SD.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

addition, CPT1, UCP3, and PPAR-δ play an important role in regulating fatty acid β-oxidation during mitochondrial transport,[28] and UCP3 can control the ROS production.[29] A current study showed that Maca (*Lepidium meyenii Walp*.) played a crucial role in alleviating physical fatigue by up-regulating mitochondrial biogenesis and function and increasing antioxidant capacity.[30] In this study, dietary Nar supplementation increased the ATP and COX III contents and COX activity and CPT1, UCP3, and PPAR-δ mRNA levels, while reduced the level of ROS and MDA in liver and GAS muscle. Nrf2/Keap1 signaling is a master regulator of antioxidant defense system by activating the transcription of antioxidant enzymes genes.[31] In this study, dietary Nar supplementation increased the activities of T-SOD, GPX, and CAT and the mRNA levels of *SOD1*, *SOD2*, *CAT*, *GPX*, and *Nrf2*, while reduced the *Keap1* mRNA level in liver and GAS muscle of mice. Together, data showed that Nar might promote the antifatigue capacity of mice by improving mitochondrial function and fatty acid β-oxidation, decreasing oxidative stress, and preventing lipid peroxidation.

It has been reported that fatigue is associated closely with inflammatory cytokine production such as TNF-α.[32] TNF-α was reported to induce the syncytin-1 (high expression in response to muscle damage and inflammation) expression, and ultimately resulted in the decrease of ASCT1 (syncytin-1 receptor) expression.[32] Furthermore, the increase of iNOS level under inflammatory conditions can also inhibit the expression of ASCT1.[33] The data indicated that Nar increased the *ASCT1* mRNA expression and decreased the *syncytin-1*, *TNF-α*, and *iNOS* mRNA levels in GAS muscle of mice. A previous study found that Nar modulated the antigen (lipopolysaccharide or *Brucella abortus*)-induced chronic fatigue in mice by attenuating the TNF-α level.[34] Furthermore, a recent study found that dietary supplementation with quercetin promoted antifatigue capacity by decreasing muscle damage and inflammation-related gene expression and enhancing muscle function.[21] Together, these results indicated that Nar improved the fatigue capacity by decreasing muscle damage and inflammation.

Based on weight-loaded and non-loading swimming tests as well as experimental analysis, this study showed that Nar had antifatigue effects. In addition, this study provided new evidence for the antifatigue mechanism of Nar. Nar improved antifatigue function of mice might be via prolonging the swimming exhaustion time, decreasing the accumulation of metabolites and enhanced energy storage, improving antioxidant capacity and mitochondrial function, and reducing muscle damage and inflammation. In the future, the antifatigue molecular mechanisms of Nar need to be further studied.

Conclusions

In summary, this study demonstrated that Nar had antifatigue effects. This study suggested that Nar might be developed as a functional food or natural drugs having an antifatigue effect.

AUTHORS' CONTRIBUTIONS

XC and ZH designed the study and wrote the manuscript. YX did the experiments and analyzed the data. GJ, HZ, and GL provided the technical support.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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