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## The rapid effects of eicosapentaenoic acid (EPA) enriched phospholipids on alleviating exercise fatigue in mice

 Cheng-Cheng Wang,<sup>a</sup> Hao-Hao Shi,<sup>a</sup> Ling-Yu Zhang,<sup>a</sup> Lin Ding,<sup>a</sup> Chang-Hu Xue,<sup>ab</sup> Teruyoshi Yanagita,<sup>c</sup> Tian-Tian Zhang  <sup>\*a</sup> and Yu-Ming Wang  <sup>ab</sup>

It has been reported that docosahexaenoic acid/eicosapentaenoic acid (DHA/EPA) and phospholipids (PLs) play an important role in alleviating exercise fatigue. However, the difference of DHA and EPA in ameliorating exercise fatigue is still unclear. Furthermore, the comparative study about DHA/EPA-PLs and nonpolar DHA/EPA on exercise fatigue has not been reported. In the present study, the effects of DHA and EPA on exercise fatigue was firstly compared by conducting an exhaustion test, and the results showed that triglyceride (TG) with high ratio of EPA had a more significant effect on alleviating exercise fatigue than TG with a low ratio of EPA in mice. Therefore, eicosapentaenoic acid–ethyl ester (EPA–EE) and EPA–PL were then selected to compare the rapid effects of polar and nonpolar DHA/EPA on exercise fatigue in mice by a weight-loaded swimming exhaustion test. A single intake of EPA–PL but not EPA–EE significantly alleviated exercise fatigue in mice by increasing the lactic acid recycling rate as well as inhibiting glycogen consumption and muscle injury, suggesting that EPA–PL exhibited a rapid effect on alleviating exercise fatigue. The study might represent a potential novel candidate or targeted dietary pattern for alleviating exercise fatigue.

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## 1. Introduction

Exercise fatigue is the transient inability to maintain optimal exercise performance caused by intense exercise loading and inadequate rest, and results in a negative impact on physiological function, such as immune, endocrine, antioxidant system, nervous system and so on.<sup>1–4</sup> Epidemiological data suggested a high prevalence of fatigue induced by physical exertion in laborers that disturbs health and life quality.<sup>5</sup> However, most people who suffer from fatigue symptoms induced by physical exertion would not take the initiative to lighten the burden of work. Therefore, it is an urgent mission to find a targeted dietary patterns or natural bio-activator for alleviating exercise fatigue.

Increasing evidence has indicated the anti-fatigue effect of  $\omega$ -3 long chain polyunsaturated fatty acids ( $\omega$ -3 LCPUFA), including docosahexaenoic acid (DHA; 22 : 6  $\omega$ -3) and eicosapentaenoic acid (EPA; 20 : 5  $\omega$ -3). Double-blind studies suggested that fish oil supplementation inhibited muscle damage

and alleviated delayed onset muscle soreness (DOMS) induced by exercise overloading in untrained individuals.<sup>6,7</sup> Animal studies reported that  $\omega$ -3 LCPUFA could elicit muscle membrane DHA enrichment, alleviate DOMS, and improve regenerative capacity of skeletal muscle cells, thereby benefiting exercise performance<sup>8,9</sup> Moreover, studies have shown that there was a highly significant relevance between dietary and/or muscle levels of  $\omega$ -3 LCPUFA and exercise performance.<sup>10</sup> Notably, several studies found that DHA and EPA exhibited different efficacy in alleviating cardiopathy, mental health disorders, etc.<sup>11,12</sup> However, the difference of DHA and EPA in ameliorating exercise fatigue is still unclear. Furthermore, the rapid effect of DHA/EPA on exercise fatigue has not been reported yet.

Additionally, emerging evidence suggested that phospholipids (PLs), such as phosphatidylcholine (PC) and phosphatidylserine (PS), played an important role in alleviating exercise fatigue.<sup>13</sup> Double-blind studies showed that PLs could reduce the diastolic blood pressure and cardio-ankle vascular index, suppress the severity of stress responses to exercise, thereby enhancing the exercise capacity.<sup>14</sup> Interestingly, marine-derived DHA/EPA esterified to phospholipids (DHA/EPA-PLs) exhibit the structural characteristics of DHA/EPA and PLs.<sup>15</sup> It has been reported that DHA/EPA-PLs had better effects than terrestrial PLs or nonpolar DHA/EPA enriched fish oil, such as triacylglycerol (TG) and ethyl ester (EE) forms, on improving neurodegenerative diseases, metabolic syndrome and so on.<sup>16,17</sup>

<sup>a</sup>College of Food Science and Engineering, Ocean University of China, No.5 Yushan Road, Qingdao 266003, P. R. China. E-mail: [zhangtiantian@ouc.edu.cn](mailto:zhangtiantian@ouc.edu.cn); [wangyuming@ouc.edu.cn](mailto:wangyuming@ouc.edu.cn); Fax: +86 0532 82032468; Tel: +86 0532 82032597

<sup>b</sup>Laboratory for Marine Drugs and Bioproducts of Qingdao National Laboratory for Marine Science and Technology, Qingdao, Shandong Province, P. R. China

<sup>c</sup>Laboratory of Nutrition Biochemistry, Department of Applied Biochemistry and Food Science, Saga University, Saga 840-8502, Japan



Le *et al.* reported that a 9 week DHA-PL enriched supplementation improved endurance exercise capacity and skeletal muscle mitochondrial function in adult rats.<sup>18</sup> However, the comparative study about DHA/EPA-PLs and nonpolar DHA/EPA on exercise fatigue has not been reported.

In the present study, running exhaustion test were firstly used to compare the effects of DHA and EPA on exercise fatigue and the results showed that TG with high ratio of EPA had better effects on alleviating exercise fatigue than TG with low ratio of EPA in mice. Therefore, EPA-EE and EPA-PL were selected to evaluate the rapid effects of polar and nonpolar DHA/EPA on exercise fatigue in mice by weight-loaded swimming exhaustion test.

## 2. Materials and methods

### 2.1 Preparation and determination of DHA/EPA

DHA enriched phospholipids and EPA enriched phospholipids were prepared from squid (*Sthenoteuthis oualaniensis*) roe and sea cucumber according to the previous method, respectively.<sup>16</sup> In brief, total lipids were extracted by the modified method of Folch,<sup>19</sup> and then phospholipids were separated by the previous method.<sup>16</sup> The composition of DHA/EPA-PLs was confirmed by high-performance liquid chromatography-evaporative light scattering detector (HPLC-ELSD) analysis, and the purity of phospholipids was more than 90%. EPA-EE (purity > 90%) was purchased from Sinomega Biotechnology Co., Ltd. (Zhoushan, China), and fish oil (DHA/EPA-TG) and alga oil (DHA-TG) were purchased from Weihai Boow Foods Co., Ltd. (Weihai, China). Fatty acid composition of each sample was determined and the result was shown in Table 1.

### 2.2 Animals and treatments

Mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China), and provided with food and water *ad libitum* in a room with a 12 h/12 h light/dark cycle, 23 ± 2 °C constant temperature and 60 ± 10% relative humidity.

Table 1 Main fatty acid compositions of different DHA/EPA supplements<sup>a</sup>

Fatty acids (%)	DHA/EPA-TG	DHA-TG	DHA/EPA-PL	EPA-EE	EPA-PL
C16 : 0	3.14	25.4	23.5	—	4.32
C16 : 1	1.00	—	—	4.52	5.39
C18 : 0	4.12	—	3.42	4.32	15.8
C18 : 1	12.9	6.22	11.5	1.78	7.95
C18 : 2n – 6	1.03	10.2	0.68	—	—
C18 : 3n – 3	0.643	4.52	2.21	—	—
C20 : 0	1.13	—	5.56	—	—
C20 : 1	0.234	—	—	1.55	10.6
C20 : 2	—	—	—	—	—
C20 : 3	—	—	—	—	—
C20 : 4n – 6	2.27	—	0.95	3.56	5.33
C20 : 5n – 3	34.5	—	10.6	72.3	49.2
C22 : 1	0.932	9.71	1.82	—	—
C22 : 6n – 3	24.7	42.9	32.6	9.59	0.68

<sup>a</sup> Note: “—”, none detected.

Table 2 Ingredients of the experimental diets<sup>a</sup>

Ingredients (g kg <sup>-1</sup> )	Control	DHA/EPA-TG	DHA-TG	DHA-PL
Casein	200	200	200	200
Sucrose	100	100	100	100
Cornstarch	399	399	399	399
Corn oil	50	45.8	44.2	44.2
Lard	150	137.3	132.5	132.7
Cellulose	50	50	50	50
Mineral-salt mix	35	35	35	35
Vitamin mix	10	10	10	10
L-methionine	3	3	3	3
Choline bitartrate	3	3	3	3
DHA/EPA-TG	—	16.9	—	—
DHA-TG	—	—	23.3	—
DHA-PL	—	—	—	23.1

<sup>a</sup> Note: “—”, none added.

All protocols and procedures were in accordance with the guidelines of the ethical committee of experimental animal care at College of Food Science and Engineering, Ocean University of China (Qingdao, China). After acclimatization, the experiment was performed as follows.

**2.2.1 Effects of DHA and EPA on the time to exhaustion in mice by consecutive administration.** Male mice (18–22 g) were randomly divided into four groups, including control, DHA/EPA-TG, DHA-TG and DHA-PL, and fed with modified diet by AIN-93G for 10 days. The ingredients of the experimental diets were summarized in Table 2. The running exhaustion test was performed on the tenth day.

**2.2.2 Rapid effects of EPA-EE and EPA-PL on the time to exhaustion in mice by single administration.** Male mice (18–22 g) were employed in the experiment. Swim training without load was performed during the acclimatization period. According to body weight and swimming ability, the mice were divided into three groups, including control, EPA-EE and EPA-PL. After fasting for 10 hours (denoted by 0 hour),



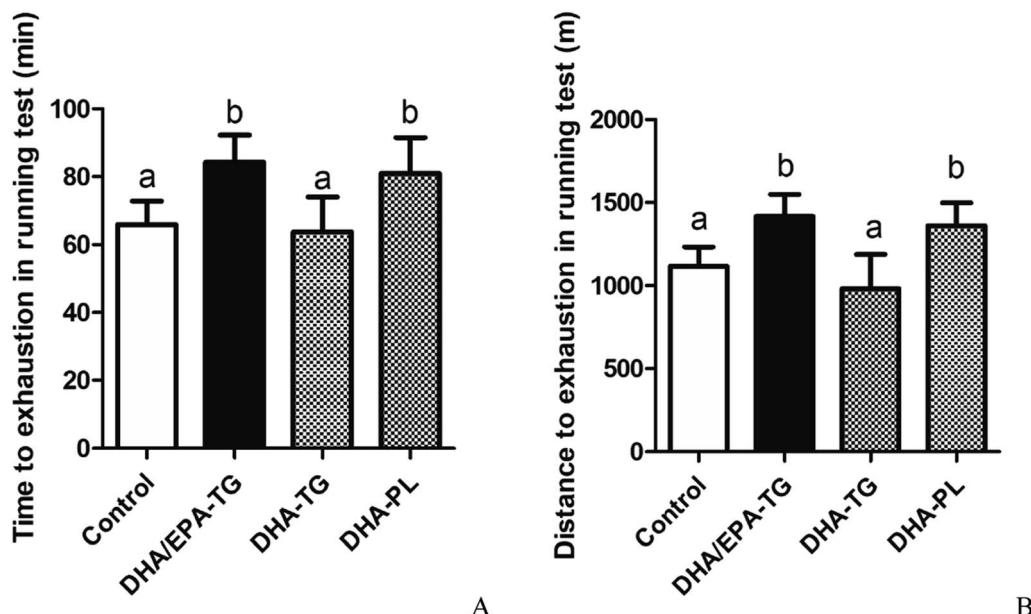


Fig. 1 Effects of different DHA/EPA formulations on the running time and distance to exhaustion in mice. (A) The time to exhaustion in running test. (B) The distance to exhaustion in running test. All data was presented as mean  $\pm$  SEM ( $n = 10$ ). Different letters indicated significant difference at  $p < 0.05$  among groups determined by ANOVA (Tukey's test).

the mice in EPA-EE and EPA-PL groups were gavaged with EPA-EE and EPA-PL emulsions ( $1000 \text{ mg kg}^{-1}$  body weight, standardized by EPA) and the control group was given equal corn oil emulsion. Weight-loaded swimming exhaustion test was performed at 2nd, 4th and 8th hour, respectively. After the last test, the mice were sacrificed rapidly or 5 minutes after rest, and the muscles and livers were collected and frozen with liquid nitrogen.

### 2.3 Running exhaustion test

A motor-driven wheel-track treadmill (YLS-10B, Shandong Academy of Medical Sciences, Jinan, China) was used in this study. All groups were suffered with an exhaustion test (25 rpm, medium difficulty coefficient). During the test, the mice stopping running were rapidly shocked by electricity (3 s, 1.5 mA) and the electric shock times were automatically recorded. When the mice rested for 30 seconds or 5 times in 5 minutes after

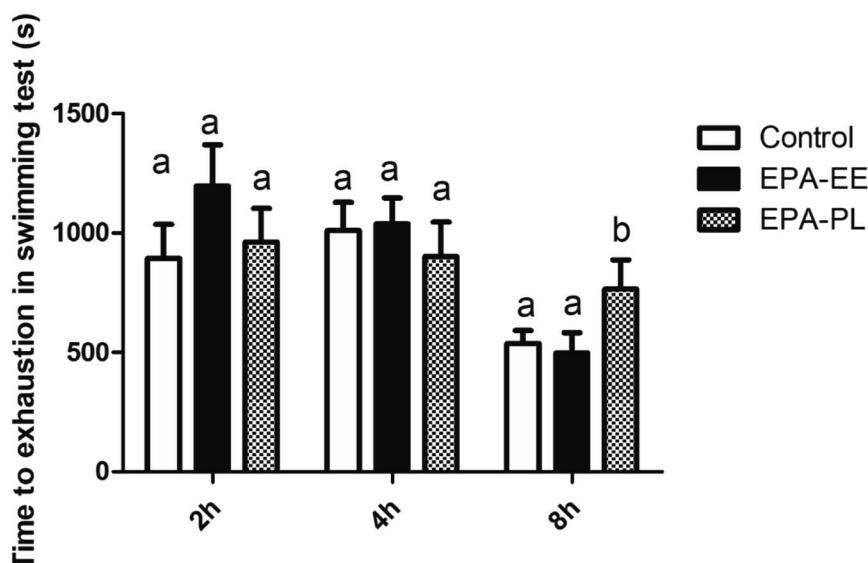


Fig. 2 Effects of single intake of different EPA on the weight-loaded swimming time to exhaustion in mice. All data was presented as mean  $\pm$  SEM ( $n = 10$ ). Different letters indicated significant difference at  $p < 0.05$  among groups determined by ANOVA (Tukey's test).

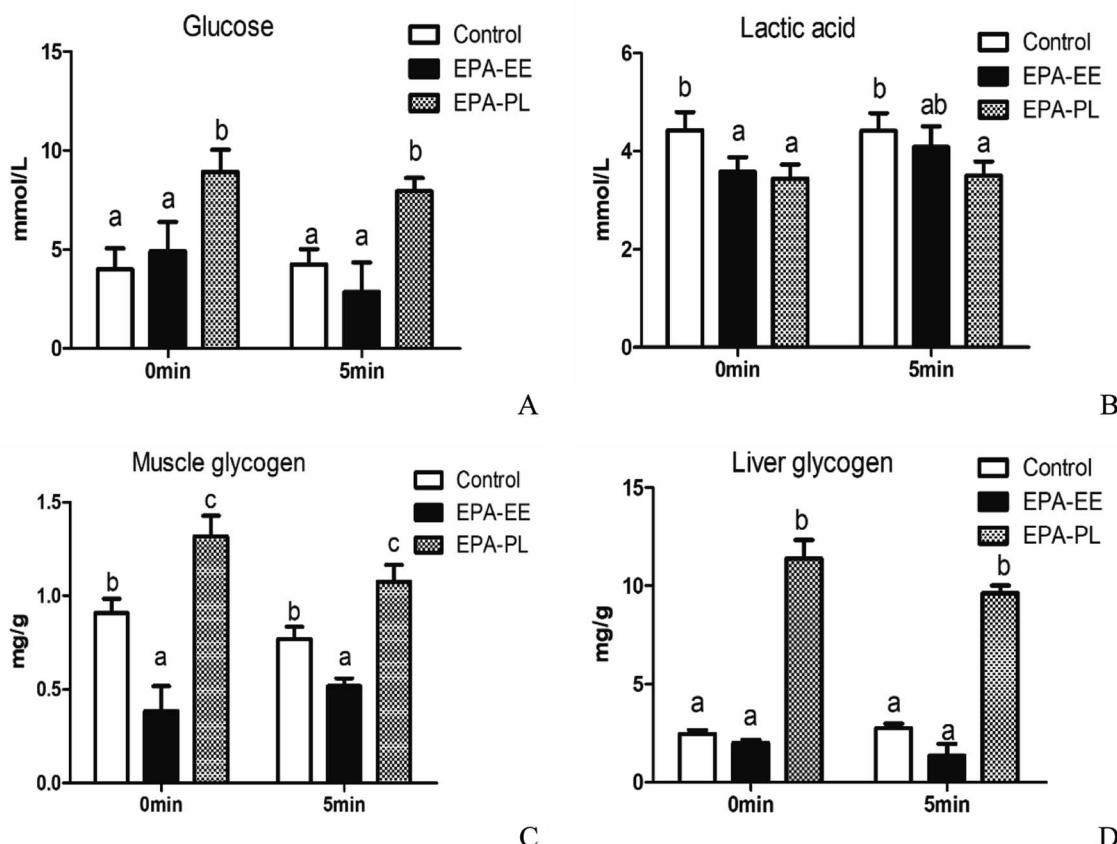


Fig. 3 Effects of single intake of different EPA on energy utilization in mice weight-loaded swimming test. (A) Glucose, (B) lactic acid, (C) muscle glycogen, (D) liver glycogen. All data was presented as mean  $\pm$  SEM ( $n = 10$ ). Different letters indicated significant difference at  $p < 0.05$  among groups determined by ANOVA (Tukey's test). \* $p < 0.05$ , significant difference between 0 min and 5 min assessed by Student's *t* test.

a long time of electric shock, they were judged to be exhausted, and the time to exhaustion was recorded.

#### 2.4 Weight-loaded swimming exhaustion test

The mice loading lead wire on tails (9% body weight) were requested to swim in the water (50 cm  $\times$  50 cm  $\times$  40 cm, 25  $\pm$  1 °C). The exhaustion time was recorded from the beginning of

swimming to the exhaustion time when the head of the mice could not float out of the water for 7 seconds.

#### 2.5 Biochemical analysis

The concentration of glucose, lactic acid, transforming growth factor beta (TGF- $\beta$ ) in serum and glycogen in muscle and liver, as well as the activity of creatine kinase (CK) in serum were

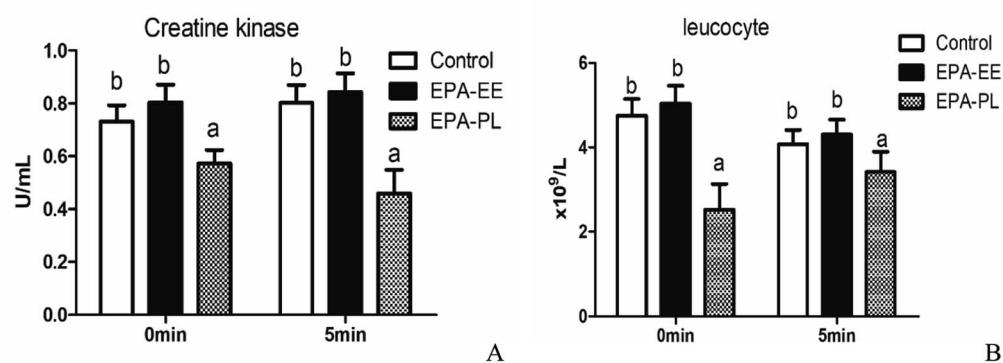


Fig. 4 Effects of single intake of different EPA on muscle injury in mice weight-loaded swimming test. (A) Creatine kinase, (B) leucocyte. All data was presented as mean  $\pm$  SEM ( $n = 10$ ). Different letters indicated significant difference at  $p < 0.05$  among groups determined by ANOVA (Tukey's test). \* $p < 0.05$ , significant difference between 0 min and 5 min assessed by Student's *t* test.

measured using assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The amounts of hemoglobin, erythrocyte and leucocyte were measured by routine blood test.

## 2.6 Statistical analysis

All data were expressed as mean  $\pm$  SEM (standard error of the mean, indicated by error bars). Differences among groups were assessed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Difference between 0 min and 5 min were assessed by Student's *t* test. Different letters indicated significant differences among groups when  $p < 0.05$ .

## 3. Results and discussion

### 3.1 Effects of DHA and EPA on the time to exhaustion in mice by consecutive administration

In order to compare the effects of different DHA/EPA formulations on exercise fatigue, the running exhaustion test were performed and the results were shown in Fig. 1. Administration of DHA/EPA-TG and DHA-PL rather than DHA-TG for 10 days significantly extended the time and distance to running exhaustion in mice. Several studies showed that administration of DHA/EPA enriched fish oil for 8–9 weeks could significantly reduce muscle fatigue with the changed fatty acid composition of membrane phospholipids in muscle.<sup>20</sup> Importantly, our previous study suggested that administration of different form of DHA/EPA for 4–14 days could significantly increase the level of hepatic and testicular DHA/EPA in mice.<sup>21</sup> In the present study, administration of DHA/EPA in different forms for 10 days did alleviate exercise fatigue in mice, which might be attributed to the changes of membrane composition and structure after  $\omega$ -3 supplementation for 10 days.

Importantly, we found that TG with higher ratio of EPA (EPA : DHA = 34.5 : 24.7) had better effects on alleviating exercise fatigue than TG with lower ratio of EPA (EPA : DHA = none detected: 42.9) in mice. It seems unreasonable and contradictory that EPA showed a superior effect than DHA on alleviating exercise fatigue as it has been reported that EPA exhibited lower concentration than DHA *in vivo*.<sup>21,22</sup> Several investigations revealed that it was an important reason for biological activity of EPA that not only EPA involved in the conversion to DHA, but also inhibiting inflammation, improving lipid metabolism and so on.<sup>23–25</sup> It might imply that EPA could play an important role in the process of substance and energy metabolism, thereby mitigating exercise fatigue, which need to be further confirmed.

Additionally, PL with lower ratio of EPA (EPA : DHA = 10.6 : 32.6) had a similar effect to TG with higher ratio of EPA (EPA : DHA = 34.5 : 24.7) on mitigating exercise fatigue in the present study. It might indicate that EPA-PL had a superior effect than EPA-EE. Le *et al.* reported that a 9 week DHA-PL enriched supplementation improved endurance exercise capacity and skeletal muscle mitochondrial function in adult rats.<sup>18</sup> Various investigations have shown that DHA-PL exhibits distinct effects in comparison with DHA-TG/EE. Our previous study found that DHA/EPA-PL was more effective than DHA/EPA-TG/EE on suppressing neuronal apoptosis in mice with Parkinson's diseases.<sup>16,26</sup> The efficiency of DHA/EPA-PL is more significant than that of the TG form in alleviating exercise fatigue, which might be attributed to the differences in absorption, distribution and incorporation of the different DHA/EPA formulations in mice. Phospholipids were efficient carriers for  $\omega$ -3 PUFA in circulation and  $\omega$ -3 PUFA enriched phospholipids had more preferential incorporation into cell membrane than  $\omega$ -3 PUFA enriched triglyceride.<sup>27,28</sup>

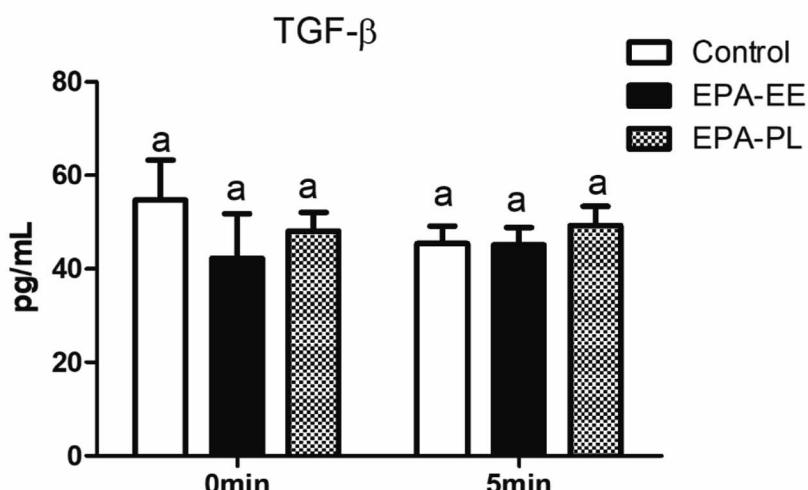


Fig. 5 Effects of single intake of different EPA on TGF- $\beta$  in mice weight-loaded swimming test. All data was presented as mean  $\pm$  SEM ( $n = 10$ ). Different letters indicated significant difference at  $p < 0.05$  among groups determined by ANOVA (Tukey's test). \* $p < 0.05$ , significant difference between 0 min and 5 min assessed by Student's *t* test.



### 3.2 Rapid effects of EPA-EE and EPA-PL on the time to exhaustion in mice by single administration

Weight-loaded swimming test with a short duration (for minutes, far less than 2 hours) was considered as a common and classical evaluation for exercise endurance. To test the hypothesis that EPA plays a role in the process of substance and energy metabolism, the weight-loaded swimming test was performed to evaluate the rapid effects of EPA-EE and EPA-PL on mitigating exercise fatigue by single administration before changing the fatty acid composition of membrane in muscle. The time to exhaustion of mice was shown in Fig. 2. Although there was no significant difference among the groups at 2nd hour and 4th hour, EPA-PL significantly increased the time to weight-loaded swimming exhaustion at 8th hour, compared with the control and EPA-EE groups. The above results showed that EPA-PL but not EPA-EE had a significant rapid effect on ameliorating exercise fatigue in mice. Jakeman *et al.* reported that adult male volunteers were immediately supplied with fish oil (750 mg EPA + 50 mg DHA or 150 mg EPA + 100 mg DHA) after muscle injury caused by jumping, and their jumping ability was significantly improved at the 48th hour after intake of fish oil.<sup>29</sup> However, the results of this study showed that intake of EPA-EE (1000 mg kg<sup>-1</sup> weight) for 8 h did not increase the time to weight-loaded swimming exhaustion in mice (the time at 48 h was not measured), which might be attributed to not only incompletely consistent exercise mode, but also the longer onset time of EPA-EE than EPA-PL. The onset time of EPA-EE/PL might be associated with the rate of digestion and absorption. Dietary lipids are mainly digested in the intestine to form fatty acids and other residual substances, such as monoglycerides or lyso-phospholipids.<sup>30</sup> Phospholipids are hydrolyzed into lyso-phospholipids and non-esterified fatty acid (NEFA) by pancreatic lipase during digestion and absorption, while ethyl esters are hydrolyzed into NEFA and ethanol by pancreatic lipase and carboxylesterase. After absorption by intestinal cells, the fatty acid and other substances are resynthesized into TG and PLs. Importantly, experiments *in vitro* showed that the rate of hydrolyzing glycerol ester by pancreatic lipase was 10–50 times higher than that of ethyl ester catalyzed by pancreatic lipase and the activity of carboxylesterase was much lower than that of pancreatic lipase.<sup>31</sup> For the

ethyl ester form, this is a less efficient process than the direct absorption of TG, leading to a worse bioavailability of the ethyl ester form compared with that of the TG form. Notably, the amphiphilic properties of PLs may affect the surface composition of fat droplets to promote the binding of hydrolases before absorption, thus PL form is more easily absorbed than the TG form.

### 3.3 Effects of EPA-EE and EPA-PL on energy utilization in mice by single administration

The mice were sacrificed rapidly or 5 minutes after the last weight-loaded swimming exhaustion test. The concentrations of glucose and lactic acid in serum, hepatic glycogen and muscle glycogen were measured and the results were shown in Fig. 3. Compared with the control group, EPA-PL inhibited the decrease of blood glucose and the increase of lactate acid caused by exercise fatigue after weight-loaded swimming exhaustion test, while EPA-EE had no significant effect on glucose content and the accumulation of lactate acid in serum (Fig. 3A and B). EPA-PL significantly increased the levels of glycogen in liver and muscle after weight-loaded swimming exhaustion test but EPA-EE had no beneficial effect (Fig. 3C and D). There was no significant difference between 0 minute and 5 minute after weight-loaded swimming exhaustion test in above parameters. Several results showed that terrestrial phospholipids and nonpolar DHA/EPA significantly inhibited the decrease of blood glucose, glycogen consumption and lactic acid accumulation, and significantly improved exercise fatigue in mice.<sup>13,20</sup> The data of biochemical analysis in the present study showed that EPA-PL significantly inhibited the decrease of blood glucose, lactate accumulation and glycogen consumption in liver and muscle, thereby reducing exercise fatigue, which was consistent with the previously reported results. During strenuous exercise, muscle consumes muscle glycogen by glycolysis and produces lactic acid that enters the liver through circulation. Although the majority of lactic acid synthesizes glycogen again in recovery by gluconeogenesis in liver, but the fraction of lactic acid can recycle by gluconeogenesis in liver during exercise. Excessive consumption of muscle glycogen causes excessive accumulation of lactic acid, which inhibit glycolysis and induce acid poisoning in muscle, thereby leading to muscle fatigue. Therefore, promoting Cori cycle (lactic acid circulates in muscle-liver-muscle) is an effective way to

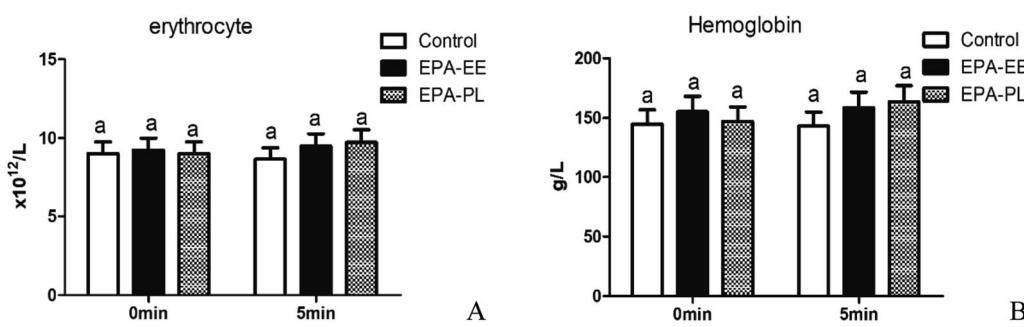


Fig. 6 Effects of single intake of different EPA on hemoglobin in mice weight-loaded swimming test. (A) Erythrocyte, (B) hemoglobin. All data was presented as mean  $\pm$  SEM ( $n = 10$ ). Different letters indicated significant difference at  $p < 0.05$  among groups determined by ANOVA (Tukey's test). \* $p < 0.05$ , significant difference between 0 min and 5 min assessed by Student's *t* test.

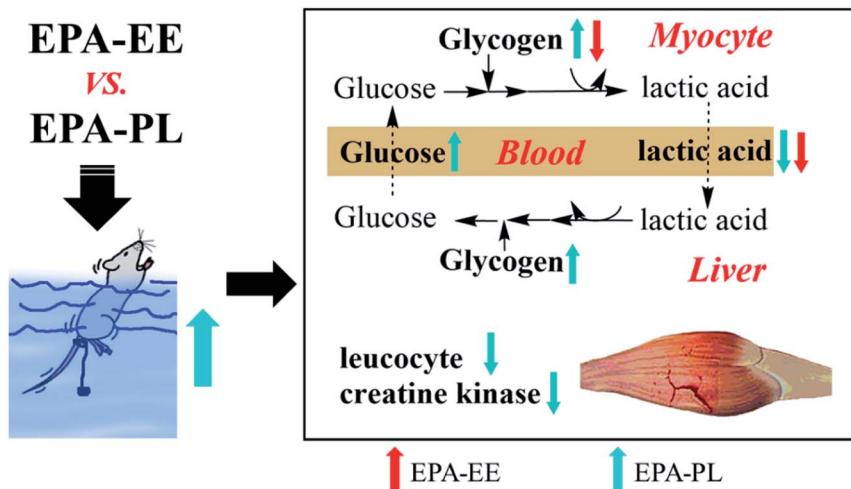


Fig. 7 The effects of EPA-EE and EPA-PL on alleviating physical fatigue induced by weight-loaded swimming in mice by single administration.

eliminate the accumulation of lactic acid in the muscle. The present study showed that single administration of EPA-PL more significantly improved lactic acid recycling rate than EE form. In addition, it might be the reason for reducing glycogen consumption that EPA-PL activated other energy metabolism pathway, such as promoting tricarboxylic acid cycle and lipid metabolism, which could also decrease the production of lactic acid.

### 3.4 Effects of EPA-EE and EPA-PL on muscle injury in mice by single administration

The number of leucocyte and the activity of CK in blood were measured to evaluate muscle injury and the results were shown in Fig. 4. Compared with the control group, EPA-PL but not EPA-EE significantly decreased the activity of CK and the amount of leucocyte after weight-loaded swimming exhaustion test. There was no significant difference for CK and leucocyte between 0 minute and 5 minute after weight-loaded swimming exhaustion test. Tinsley *et al.* showed that fish oil supplementation (6 g day<sup>-1</sup>, EPA : DHA = 5 : 1) for one week significantly improved muscle soreness and muscle injury caused by resistance training in adult women.<sup>32</sup> Peoples *et al.* suggested that intake of fish oil for 8 weeks significantly increased muscle strength in male rat using a fatigue model by perfusing the hind limbs of rats with continuous hypoxia and stimulating gastrocnemius-metatarsal-soleus muscle bundle.<sup>8</sup> The present study showed that EPA-PL significantly inhibited the activity of CK and the number of leucocytes, suggesting that EPA-PL could alleviate muscle injury induced by weight-loaded swimming exhaustion in mice, which was consistent with the reported results.

### 3.5 Effects of EPA-EE and EPA-PL on TGF- $\beta$ in mice by single administration

Increased levels of transforming growth factor  $\beta$  (TGF- $\beta$ ) in circulation and brain lead to the perception of fatigue and reduce autonomous activity. It has been reported that the significantly high level of TGF- $\beta$  was found in the brain of

animals or patients with chronic fatigue syndrome.<sup>33</sup> Besides, TGF- $\beta$  is associated with inflammation. The concentration of TGF- $\beta$  in serum was measured in mice (Fig. 5). However, exhaustion and single intake of EPA could not affect TGF- $\beta$  level in the present study. Cao *et al.* reported that DHA-PL and EPA-PL could suppress the expressions of TGF- $\beta$  activated kinase 1 in mice with chronic stress.<sup>34</sup> Shimojo *et al.* showed that EPA suppressed the expression of TGF- $\beta$  *in vitro*.<sup>35</sup> In the present study, short-term exercise fatigue could not affect the level of TGF- $\beta$ .

### 3.6 Effects of EPA-EE and EPA-PL on hemoglobin in mice by single administration

During exercise, oxygen is transported from the respiratory system to muscles and other organs by erythrocyte, and the concentration of hemoglobin reflects carrying oxygen capacity of erythrocyte. The number of erythrocyte and the concentration of hemoglobin were measured and the results were shown in Fig. 6. Unexpectedly, no significant difference among the groups was found in present study, which was presumably due to the short intervention time. Moyers *et al.* reported that  $\omega$ -3 fatty acid levels in whole blood were not related to the concentration of hemoglobin in patients with stable coronary artery disease, which could reveal the invalidity of EPA to the concentration of hemoglobin.<sup>36</sup>

## 4. Conclusions

In the present study, running exhaustion test was used to compare the effects of DHA and EPA on exercise fatigue, and results showed that administration of DHA/EPA-TG and DHA-PL rather than DHA-TG significantly extended the time and distance to running exhaustion in mice. Interestingly, TG with higher ratio of EPA had better effects on alleviating exercise fatigue than TG with lower ratio of EPA in mice. Single administration of EPA-PL but not EPA-EE significantly alleviated exercise fatigue in mice by increasing lactic acid recycling



rate as well as inhibiting glycogen consumption and muscle injury, suggesting that EPA-PL exhibited rapid effect on alleviating exercise fatigue (Fig. 7). The study might represent a potential novel candidate or targeted dietary patterns for alleviating exercise fatigue.

## Ethics statement

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of the National Institutes of Health and experiments were approved by the Animal Ethics Committee of experimental animal care at the College of Food Science and Engineering, Ocean University of China (Qingdao, China, approval no. SPXY2015012).

## Conflicts of interest

The authors declare no competing financial interest.

## Acknowledgements

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