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Effect of Erabu Sea Snake *(Laticauda semifasciata)* Lipids on the Swimming Endurance of Mice

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Key Words
Endurance · Glycogen · Lactate · *Laticauda semifasciata* · Mice · Sea snake lipids

Abstract
Background/Aim: This study was designed to investigate the effect of Erabu sea snake *(Laticauda semifasciata)* lipids on the swimming endurance of mice. ***Method:*** Twelve-week-old male Crlj: CD-1 (ICR) mice were fed one of three experimental diets containing 6% lard, fish oil or sea snake lipids for 16 weeks. Swimming exercise was conducted in an acrylic plastic tank filled with 25 cm of water maintained at 23°C. Every 4 weeks, the mice were made to perform swimming exercises with loads attached to their tails, corresponding to approximately 1 or 2% of their body weights. ***Results:*** The group fed the sea snake lipid diet exhibited significantly improved swimming endurance compared with the lard diet group ($p < 0.05$); however, this result was not observed in the fish oil diet group. In the sea snake lipid diet group, plasma and muscle lactates were significantly lower, and plasma glucose and muscle glycogen were significantly higher than in the lard diet group ($p < 0.05$). ***Conclusion:*** These results suggest that the intake of sea snake lipids enhanced the swimming endurance of the mice by delaying the accumulation of lactate during swimming exercise.

Introduction

Some food components provide enhanced endurance in animals or humans. Examples include highly branched cyclic dextrin [1], medium-chain triglycerides [2], capsaicin [3–5], (–)-hydroxycitrate [6–8], caffeine [9, 10], branched-chain amino acid [11], and conjugated linoleic acid [12].

The Erabu sea snake *(Laticauda semifasciata)*, a marine reptile, is distributed throughout the Pacific Ocean and along the coast of the South China Sea. It has been used as a folk medicine for a long time in China and Okinawa (Japan). In Japan, lipids from a fat sack in the viscera of the sea snake have also been used as a food supplement with health-promoting benefits [13]. Studies have indicated that Erabu sea snake lipids improved the learning ability of mice in a manner similar to that of fish oil, as it contains abundant amounts of n–3 polyunsaturated fatty acids (n–3 PUFAs; e.g. 20:5n–3 and 22:6n–3) [14]. A diet of these lipids also reduced plasma lipids, liver lipids, and plasma glucose in mice. In this action, this diet was more effective than a fish oil diet that contained the same percentage of 22:6n–3 [15].

In a study of anti-fatigue of snake extracts, administration of a 50% ethanol extract from the dried whole body of *Agkistrodon blomhoffii blomhoffii* Boie a venomous snake called 'Mamushi' in Japan, enhanced the swim-

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Table 1. Composition of the experimental diets (%)

	Dietary group		
	Lard	Fish oil	Sea snake lipids
Corn starch	47.8	47.8	47.8
Casein	20	20	20
Granulated sugar	15	15	15
Cellulose	5	5	5
Salt mixture	4	4	4
Vitamin mixture	2	2	2
L-Methionine	0.2	0.2	0.2
Lard	6	–	–
Fish oil	–	6	–
Sea snake lipids	–	–	6

ming endurance of rats and had an anti-fatigue effect [16]. We speculated that Erabu sea snake lipids, when used as a dietary supplement, might also enhance the endurance of mice.

Materials and Methods

Animals

Four-week-old male Crlj:CD-1 (ICR) mice were purchased from Charles River Japan (Atsugi, Japan) and housed in cages under automatically controlled conditions of temperature (24± 0.5°C), relative humidity (65±5%), and lighting (light from 07.00 to 19.00 h). All the mice were switched from laboratory chow (MF; Oriental Yeast, Tokyo, Japan), to three different experimental diets (lard, fish oil, and sea snake lipid) at 12 weeks of age. The animals had free access to the experimental diet and water until they were sacrificed. Thirty mice were randomly divided into three groups (10 in each group) with equal body weights. The mice were fed one of the three experimental diets for 16 weeks. The body weights of the mice were determined every 2 weeks. The care and treatment of the mice conformed to the guidelines for experimental animals of the National Food Research Institute, Japan.

Experimental Diets

The lard was supplied by NOF (Tokyo, Japan); the fish oil by Nippon Chemical Feed (Hakodate, Japan), and the sea snake lipids by Fuji Pharmaceutical (Tokyo, Japan). The compositions of the three experimental diets are listed in table 1. To prevent oxidative changes in fatty acid composition during storage, each experimental diet was stored below –40°C.

The fatty acid composition of each experimental diet is presented in table 2. The predominant fatty acids in all experimental diets were 16:0, 18:0, 18:1n–9, and 18:2n–6. The sea snake lipid and fish oil diets had less 18:1n–9 and 18:2n–6 and more n–3 PUFAs (e.g. 20:5n–3 and 22:6n–3) than the lard diet. The sea snake lipid diet contained less 20:5n–3 and 22:6n–3 than the fish oil diet. The lard diet had low amounts of 18:3n–3 as the only n–3 PUFA. The n–3/n–6 ratio of the fish oil diet was the highest, and that of the lard diet was the lowest of the three experimental diets.

Table 2. Main fatty acid compositions of the experimental diets (%)

	Dietary group		
	Lard	Fish oil	Sea snake lipids
Saturated fatty acid			
14:0	2.0	3.5	2.7
15:0	0.2	1.0	0.9
16:0	24.9	22.1	30.8
17:0	0.5	1.2	1.3
18:0	12.4	5.7	8.7
Monounsaturated fatty acid			
16:1n–9	0.3	0.3	0.3
16:1n–7	3.2	5.4	5.7
18:1n–9	41.7	17.7	22.7
18:1n–7	3.0	2.6	2.5
20:1n–9	0.6	1.4	0.5
Polyunsaturated fatty acid			
18:2n–6	7.8	1.6	1.4
18:3n–6	–	0.3	0.3
18:3n–3	0.4	0.5	0.4
18:4n–3	–	0.7	0.2
20:4n–6	–	1.7	1.5
20:5n–3	–	5.2	1.4
22:4n–6	–	0.2	0.9
22:5n–6	–	1.4	1.3
22:5n–3	–	1.2	1.5
22:6n–3	–	20.3	11.0
n–3/n–6	0.05	5.37	2.69

Measurement of Swimming Endurance

A forced swimming test was employed in our study to evaluate the swimming endurance of the mice fed the three experimental diets. It is commonly accepted that swimming is an effective experimental exercise model. The swimming endurance of the mice was measured in an acrylic plastic tank (26×22×29 cm) filled with water to a depth of 25 cm. The water temperature was maintained at 23°C. A soapless soap solution (1% neutral kitchen detergent; Kao, Tokyo, Japan) was used to wash the bodies and thoroughly moisten the hairs of the mice before the swimming exercise, to prevent any buoyancy differences [17, 18].

The swimming tests were carried out every 4 weeks during the feeding trial. Prior to each test, swimming training was carried out for 15 min with no loads attached to the tails of the mice. Subsequently, the swimming times of the mice supporting 1% loads were determined on the following day, and those supporting 2% loads were determined on the day after that. The swimming time to exhaustion and increase in swimming time were used as indices of the swimming endurance of the mice. The mice were as-

sessed as exhausted when they failed to rise to the surface of the water to breathe within a 7-second period; at this point they were immediately rescued from the tank [19]. The swimming test was carried out between 11.00 and 17.00, in order to avoid circadian variations in physical activity; it has been confirmed that the minimal variation in endurance occurs in mice at these times [19].

Endurance training has been demonstrated to increase the endurance of skeletal muscles [20, 21]. In this study, the 15-min swimming training was carried out only once in order to accustom the mice to swimming prior to each test, thus eliminating any possibility of influencing change in swimming times that might have been evident with increased swimming training.

Sample Collection and Processing

At the end of the feeding trial and after 3 days of physical recovery from the fatigue of the swimming test, nonfasted mice were subjected to a further swimming test for 5 min with loads that were 1% of their body weights attached to their tails. Following this test, the mice were immediately sacrificed by dislocation of the neck. Blood samples were collected from the inferior vena cava with a heparinized syringe and put into ice-cold tubes. The muscle tissues from the hind legs of the mice were then removed and stored at –30°C for the measurements of lactate and glycogen. A 0.2-ml blood sample was immediately deproteinized with perchloric acid (0.6 mol/l) and centrifuged at 3,000 *g* for 10 min. The plasma lactate in the supernatant was determined. The residual blood sample was centrifuged at 900 *g* for 15 min, and the supernatant stored at –30°C for the measurements of plasma glucose and non-esterified fatty acid (NEFA) concentrations.

Analysis of Biochemical Parameters

Plasma glucose was determined by the glucose oxidase method and NEFA by the acyl-CoA oxidase method, using commercial kits (Glucose C II test Wako and NEFA C-test Wako; Wako Pure Chemical Industries, Osaka, Japan). The plasma and skeletal muscle L-(+)-lactates were measured using an enzymatic analysis with lactate dehydrogenase and NAD^+ [22]. Skeletal muscle glycogen was determined using an enzymatic method with amyloglucosidase [23].

Statistical Analysis

Data were expressed as means ± SE. Comparisons of swimming endurance and biochemical parameters among the lard, fish oil, and sea snake lipid groups were assessed using one-way analysis of variance (ANOVA) and Duncan's multiple range test using the Statistica statistical program package (StatSoft, Tulsa, Okla., USA). A level of $p < 0.05$ was used as the criterion for statistical significance.

Results

Body Weights and Food Intake

Time courses of body weights and food intake of the mice fed the three experimental diets are presented in figure 1. No significant differences in the body weights among the three diet groups were observed at each time point ($p > 0.05$). The dietary intake increased in the first 2 weeks of feeding, decreased from the 2nd to the 5th week, and then reached a plateau after the 6th week. The average dietary intake in each group was: lard diet group, 4.1 g/day; fish oil diet group, 3.9 g/day, and sea snake lipid diet group, 4.1 g/day.

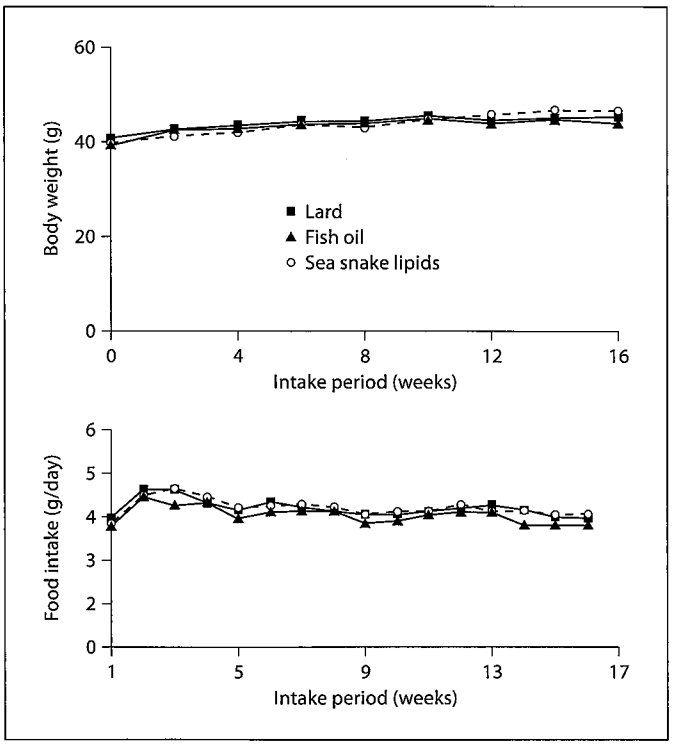

Fig. 1. Time courses of body weights and food intake of mice.

Swimming Endurance of Mice with 1% Loads Attached

The swimming endurance data of the mice supporting constant loads corresponding to 1% of their body weights in the three diet groups are plotted in figure 2. The increases in the swimming times of the mice fed the sea snake lipid diet were significantly higher at weeks 12 and 16 than those of the lard and fish oil diet groups ($p < 0.05$). They were not significantly different between the fish oil and lard diet groups ($p > 0.05$).

Analysis of the actual swimming times revealed that although the improvement in the sea snake lipid diet group was greater than that of the lard diet group, this difference was not statistically significant at weeks 12 and 16 ($p > 0.05$). However, the swimming times in the sea snake lipid diet group were significantly longer than those in the fish oil diet group at these time points ($p <$

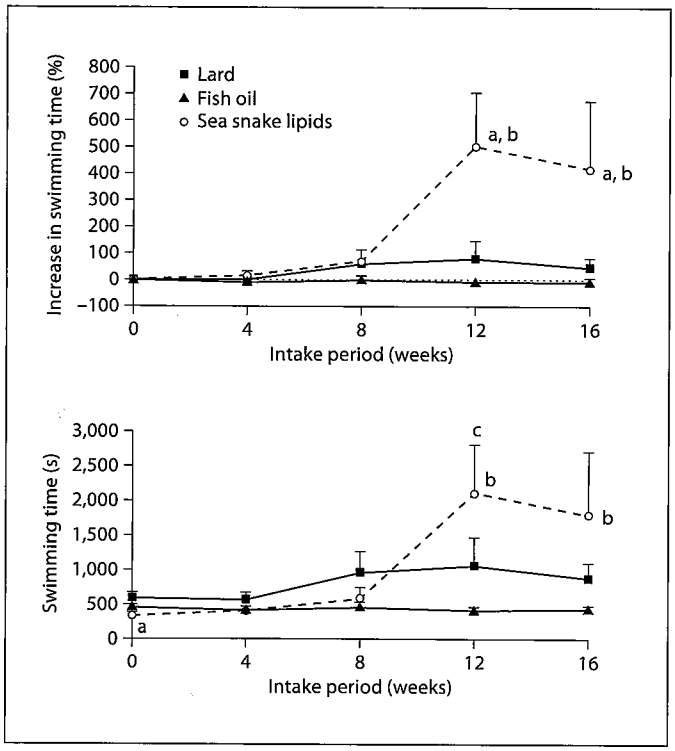

Fig. 2. Effects of lard, fish oil, and sea snake lipids on swimming endurance of mice (1% loads). Means ± SE. [a] $p < 0.05$ vs. the lard diet group, [b] $p < 0.05$ vs. the fish oil diet group, [c] $p < 0.05$ vs. swimming time at week 0.

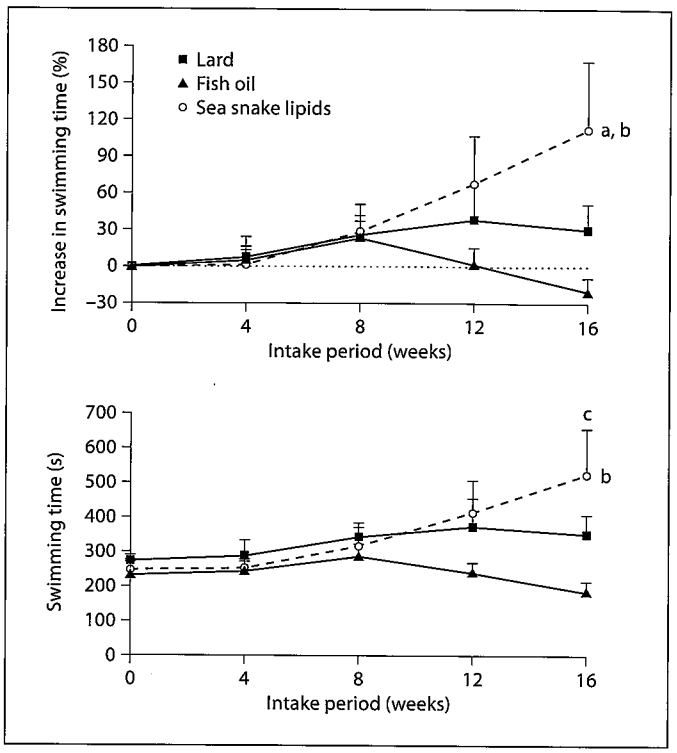

Fig. 3. Effects of lard, fish oil, and sea snake lipids on swimming endurance of mice (2% loads). Means ± SE. [a] $p < 0.05$ vs. the lard diet group, [b] $p < 0.05$ vs. the fish oil diet group, [c] $p < 0.05$ vs. swimming time at week 0.

0.05). No significant differences between the swimming times of the fish oil and those of the lard diet groups ($p > 0.05$) were observed. The initial swimming times of the sea snake lipid diet group were significantly shorter than those of lard diet group at week 0 ($p < 0.05$). Moreover, the swimming times of the sea snake lipid diet group were significantly longer at week 12 than at week 0 ($p < 0.05$). No significant increase at week 12 was observed in either the lard or fish oil diet groups ($p > 0.05$).

Swimming Endurance of Mice with 2% Loads Attached

The swimming endurance data of mice which supported constant loads corresponding to 2% of their body weights in the three diet groups are plotted in figure 3. The increases in the swimming times of the sea snake lipid diet group at week 16 were significantly greater than those of the lard and fish oil diet groups ($p < 0.05$). No significant differences in the increases in swimming times between the fish oil and lard diet groups were observed at any time point ($p > 0.05$).

Similar to the pattern seen with the 1% loads, the actual swimming times of the mice supporting 2% loads were not significantly different between the sea snake lipid and lard diet groups at week 16 ($p > 0.05$). Swimming times at week 16 were significantly longer in the sea snake lipid diet group than in the fish oil diet groups ($p < 0.05$). Again, no significant differences in the swimming time between the fish oil and lard diet groups were observed at any time point ($p > 0.05$). Moreover, no significant difference was observed in the swimming times at week 0 among the three diet groups ($p > 0.05$). The swimming times of the sea snake lipid diet group were significantly longer at week 16 compared with week 0 ($p < 0.05$); however, this result was not observed in the fish oil and lard diet groups.

Biochemical Parameters

The lactate and glycogen levels in plasma and skeletal muscle after 5 min of swimming exercises are presented in figure 4. Both plasma and skeletal muscle lactate levels were significantly lower in the sea snake lipid diet group

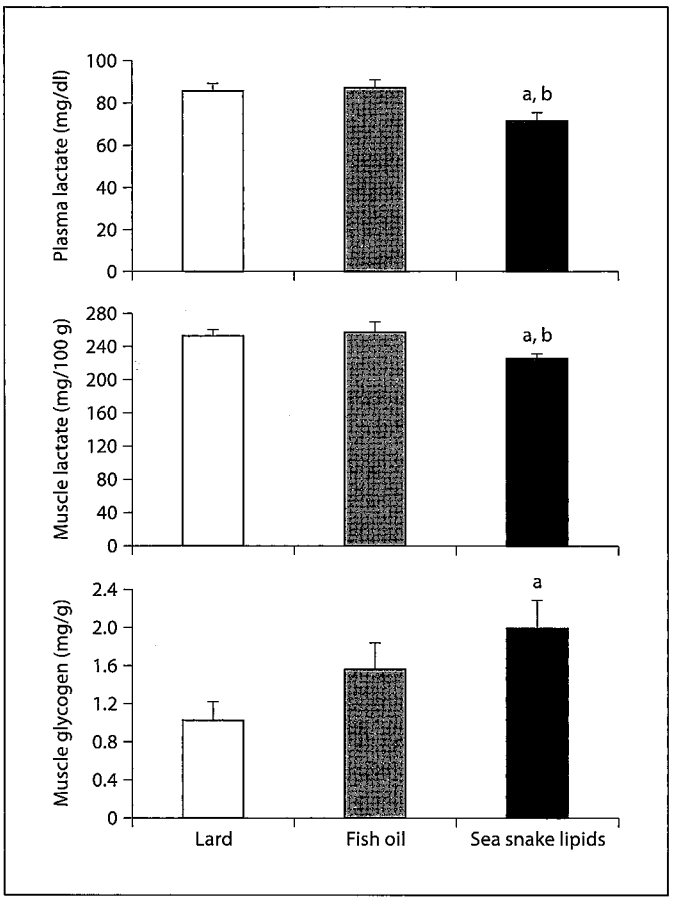

Fig. 4. Effects of lard, fish oil, and sea snake lipids on lactate and glycogen levels in plasma and skeletal muscle in mice, supporting loads that were 1% of their body weights, subjected to swimming for 5 min. Means ± SE. [a] $p < 0.05$ vs. the lard diet group, [b] $p < 0.05$ vs. the fish oil diet group.

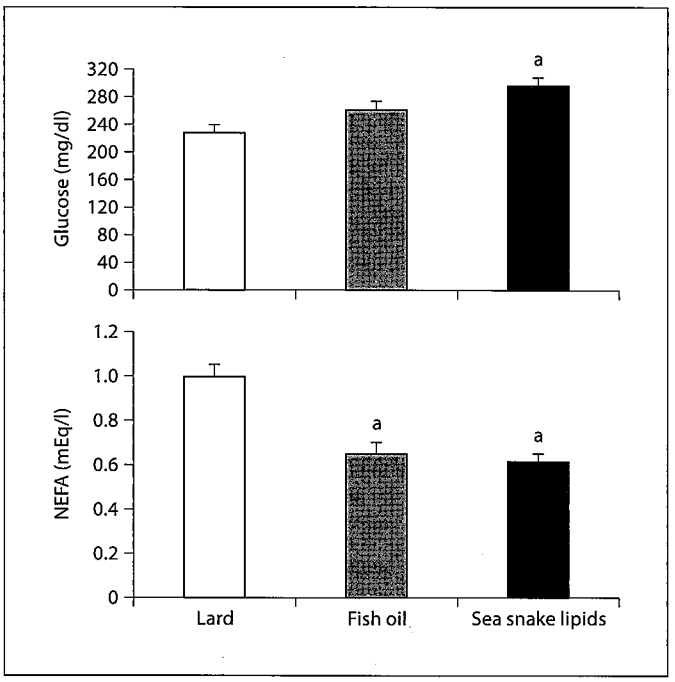

Fig. 5. Effects of lard, fish oil, and sea snake lipids on glucose and NEFA levels in plasma in mice, supporting loads that were 1% of their body weights, subjected to swimming for 5 min. Means ± SE. [a] $p < 0.05$ vs. the lard diet group.

than in the lard and fish oil diet groups ($p < 0.05$). No significant differences between these levels were observed in the lard diet group and the fish oil diet group ($p > 0.05$). The skeletal muscle glycogen levels were significantly higher in the sea snake lipid diet group than in the lard diet group ($p < 0.05$), but those in the fish oil diet group revealed no significant differences from those in the lard or sea snake lipid diet groups ($p > 0.05$).

The glucose and NEFA concentrations in the plasma after 5 min of swimming exercise are depicted in figure 5. The plasma glucose concentrations of the sea snake lipid diet group were significantly higher than those of lard diet group ($p < 0.05$), but again no significant differences between the fish oil diet group and lard or sea snake lipid diet groups ($p > 0.05$) were evident. In contrast, the plasma NEFA concentrations in the fish oil and sea snake lipid diet groups were significantly lower than those in the lard diet group ($p < 0.05$). For this parameter, no significant differences between the sea snake lipid and fish oil diet groups ($p > 0.05$) were observed.

Discussion

In this study, we investigated the effect of sea snake lipids on the swimming endurance of mice. The increases in swimming times of the sea snake lipid diet group compared with those of lard and fish oil diet groups indicated greater endurance in mice following this diet. Furthermore, only in this diet group did the swimming times of mice supporting 1 and 2% loads significantly increase compared to the times of week 0. However, it was apparent that the increased swimming endurance was acquired after mice were fed the sea snake lipid diet for 12 weeks or more. Swimming endurance was enhanced by the intake of the sea snake lipid diet without a marked difference in body weight compared with the mice receiving the fish oil and lard diets, suggesting that improved

endurance is mainly due to the effect of sea snake lipids and not a secondary effect caused by the difference in buoyancy of body fat.

Lactate is generated during anaerobic metabolism, and its production is associated with fatigue [24]. Lower levels of lactates in both plasma and skeletal muscle were observed in the sea snake lipid diet group than in the lard and fish oil diet groups, suggesting that the mice receiving sea snake lipids were less fatigued and therefore had prolonged swimming times. These results were supported by those of Fushiki et al. [2] who observed enhanced swimming endurance and lower serum lactate levels in mice following chronic administration of a diet containing medium-chain triglycerides compared with those fed a long-chain triglyceride diet. Similarly, intakes of *Anoectochilus formosanus* [25], *Paecilomyces japonica, Grifola frondosa* [26], or green tea extract [27] resulted in a significant improvement of endurance and in lower lactate concentrations compared with control groups. It was suggested that intakes of these components may attenuate production and/or enhance clearance of lactate during exercises, and the sea snake lipid intake may have the same effect.

Skeletal muscle glycogen is an essential fuel source for endurance exercise, and 'glycogen sparing' enables prolongation of such exercise [28, 29]. In the present study, the levels of plasma glucose and muscle glycogen after 5 min of swimming exercise were significantly higher in the mice fed the sea snake lipid diet than in those fed the lard diet. This result suggests that the mice receiving sea snake lipids may have decreased depletion of plasma glucose and muscle glycogen compared with the mice receiving lard during swimming exercise. Plasma and skeletal muscle lactate levels were significantly lower in the sea snake lipid diet group compared with those in the lard and fish oil diet groups, suggesting more aerobic metabolism in these animals and, hence, less depletion of glycogen stores in the mice fed the sea snake lipid diet.

Plasma NEFA is also a major fuel source for skeletal muscle metabolism during prolonged exercise, and mobilization of NEFA for utilization as energy can spare glycogen and improve endurance [30]. Long-term intakes of green tea extract [27] or oral administration of capsaicin before exercise [31] increased endurance by increasing fatty acid utilization in mice or rats; in these studies, the plasma NEFA concentrations were significantly higher in the animals of the test groups than those of the control groups after 30 min of swimming exercise. However, in our study, the plasma NEFA concentrations were significantly lower in the sea snake lipid diet group, where the swimming endurance was enhanced, than in the lard group after 5 min of swimming exercise. Therefore, it appears that a switch to aerobic metabolism that involves lipid utilization does not completely explain the increased endurance of the mice in the current experiments. It is possible that a long-term intake of sea snake lipids promoted gluconeogenesis, but further work is required to elucidate the mechanisms by which these changes in basic aerobic/anaerobic metabolism are achieved.

Other researchers have reported a decrease in plasma NEFA levels following an intake of DHA (22:6n–3) in KK-A^y mice [32]. Both the sea snake lipids and fish oil contained abundant n–3 PUFAs (e.g. 22:6n–3), and other reports have indicated a significant reduction in plasma and liver lipids in mice following an intake of sea snake lipid diet compared with an intake of lard diet [15]. Therefore, the low NEFA levels in the sea snake lipid and fish oil diet groups may have been caused by the suppression of lipid metabolism by n–3 PUFAs. Intake of a diet enriched with n–3 PUFAs did not improve endurance in animal or human experiments [33, 34], suggesting that n–3 PUFAs may make little or no contribution to improving endurance. It is possible that other specific components contained only in the sea snake lipids and not related to aerobic metabolism influence the swimming endurance of mice, but such factors and their mechanism of action are still to be identified.

In summary, the long-term intake of a diet containing 6% sea snake lipids improves the swimming endurance of mice. This effect is accompanied by an attenuation of lactate production and/or enhancement of lactate clearance. Further study is needed to clarify the complex mechanisms by which the endurance is enhanced. Although the efficacy of sea snake lipids in improving endurance has not yet been confirmed in human studies, our results suggest that sea snake lipids as food additives may be beneficial to enhance physical capacity in humans.

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