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

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Summary The effect of Erabu sea snake (*Laticauda semifasciata*) lipids on the swimming endurance was investigated in aged mice. Fifty three-week-old male Crlj:CD-1 (ICR) mice were fed one of three experimental diets containing either 6% lard, 6% fish oil, or 6% sea snake lipids for 16 wk. The swimming exercise was carried out in an acrylic plastic tank filled with 25 cm of water maintained at 23°C. Swimming times to exhaustion were measured with a load of 2% of their body weights attached to the tails of the mice. The swimming times to exhaustion of the group that were fed the sea snake lipid diet tended to be longer than those of the lard diet group, and were significantly improved compared with the fish oil diet group ($p<0.05$). The plasma and muscle lactate levels were significantly lower in the sea snake lipid diet group than in the lard and fish oil diet groups ($p<0.05$). The liver glycogen and plasma glucose levels of the sea snake lipid diet group did not differ markedly from those of the lard diet group ($p>0.05$), and were significantly higher than those of the fish oil diet group ($p<0.05$). These results suggest that an intake of sea snake lipids but not the fish oil, which is also rich in *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), is useful for improving the swimming endurance of aged mice by attenuating lactate production and/or enhancing lactate clearance during swimming exercise, and the *n*-3 PUFAs contained in the sea snake lipids did little or nothing for this improved endurance.

Key Words sea snake lipids, *Laticauda semifasciata*, endurance, lactate, aged mice

Numerous animal studies have reported that dietary supplementation with food, such as chicken breast extracts (*1*), conjugated linoleic acids (*2*), green tea extract (*3*), octacosanol (*4*), or oxaloacetate precursors and carnitine (*5*), enhanced endurance. However, those studies focused on young rats or mice. No results of studies of dietary supplementation and endurance in healthy aged animals have been reported.

The Erabu sea snake (*Laticauda semifasciata*), a marine reptile, exists throughout the Pacific Ocean and along the coast of the South China Sea. The lipids, extracted from a fat sack in the viscera of the sea snake, have been used as a food supplement to promote health in Japan (*6*). The lipids contain abundant amounts of *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), such as eicosapentaenoic acid (EPA; 20:5*n*-3) and docosahexaenoic acid (DHA; 22:6*n*-3). Shirai et al. (*7*) reported that Erabu sea snake lipids improved the learning ability of mice in a manner similar to that of fish oil. A sea snake lipid diet lowered plasma and liver lipids more effectively than a fish oil diet in mice in biochemical analyses, even though they contained the same percentages of 22:6*n*-3 (*8*).

Administration of a 50% ethanol extract from the dried body of *Agkistrodon blomhoffii blomhoffii* Boie, a venomous snake known as "Mamushi" in Japan, enhanced the swimming endurance of rats in a study of the anti-fatigue effects of snake extracts (*9*). It indicated that certain components in the snake species improved the endurance of experimental animals. We previously demonstrated that intake of a sea snake lipid diet enhances the swimming endurance of 12-wk-old (young) mice (*10*). The present study investigates the effects of long-term dietary intake of sea snake lipids on the swimming endurance of healthy aged mice in a weight-loaded forced swimming test.

MATERIALS AND METHODS

Animals. Four-week-old male Crlj:CD-1 (ICR) mice were purchased from Charles River Japan Inc. (Atsugi, Kanagawa, Japan) and housed in suspended stainless-steel cages with wire mesh bottoms. The animal room was kept at a temperature of 24±0.5°C and a relative humidity of 65±5%. Room lighting consisted of 12-h periods of light and dark. All mice were switched from laboratory chow, MF (Oriental Yeast Co. Ltd., Tokyo, Japan), to three different experimental diets (lard, fish

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oil, and sea snake lipids) at 53 wk of age. The animals had free access to the experimental diet and water until they were sacrificed. Fifty-one aged mice were randomly divided into three groups (17 in each group) with similar body weights and swimming times in a swimming test. Two cages were used for each diet group; each group was divided into two subgroups to feed (8 or 9 in each cage). The mice were fed one of the three experimental diets for 16 wk. The care and treatment of the mice conformed to the guidelines for experimental animals of the National Agriculture and Food Research Organization (NARO), Japan.

Experimental diets. The lard was supplied by NOF Co., Ltd. (Tokyo, Japan); the fish oil by Nippon Chemical Feed Co., Ltd. (Hakodate, Japan); and the sea snake lipids by Fuji Pharmaceutical Inc. (Tokyo, Japan). Each experimental diet contained 6% oil or fat and the following other components: 47.8% corn starch, 20% casein, 15% granulated sugar, 5% cellulose, 4% salt mixture, 2% vitamin mixture, and 0.2% L-methionine. Each experimental diet was stored below −40°C to prevent oxidative changes in the fatty acid composition during storage.

The main fatty acid composition of each experimental diet is presented in Table 1. The percentages of 18:0, 18:1*n*-9, and 18:2*n*-6 were lower and those of the *n*-3 PUFAs, such as 20:5*n*-3 and 22:6*n*-3, were higher in the fish oil and sea snake lipid diets than in the lard diet. The sea snake lipid diet contained less 20:5*n*-3 and 22:6*n*-3 and more 16:0, 18:0, and 18:1*n*-9 than the fish oil diet.

Swimming endurance. It is commonly accepted that swimming is a good experimental exercise model. The swimming times to exhaustion of aged mice were measured in an acrylic plastic tank (26×22×29 cm) filled with water to a depth of 25 cm and maintained at a temperature of 23°C. A soapless solution (1% neutral kitchen detergent, Kao Co. Ltd., Japan) was used to wash their bodies and thoroughly moisten their hairs before the swimming exercise to prevent buoyancy differences among the aged mice (*11*, *12*).

Table 1. Main fatty acid compositions of the experimental diets (%).

	Dietary group		
	Lard	Fish oil	Sea snake lipids
SFA			
16:0	25.8	21.0	29.9
18:0	12.7	5.4	8.5
MUFA			
16:1	3.3	5.7	5.7
18:1*n*-9	40.8	16.7	22.4
18:1*n*-7	2.8	2.5	2.3
PUFA			
18:2*n*-6	8.2	1.6	1.4
20:4*n*-6	—	1.6	1.6
20:5*n*-3	—	5.4	1.5
22:6*n*-3	—	19.3	11.8

SFA: saturated fatty acid, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid.

The swimming tests were carried out before and after the feeding trials. A swimming training session was carried out for 10 min on the day prior to each trial with no loads attached to the tails of the aged mice. The swimming times to exhaustion of aged mice supporting 2% loads were determined twice at the same time point with 2 d of rest between the two determinations before or after the feeding trials. The mice dying of old age during the feeding trial were eliminated after the feeding trial, and the swimming times of the remaining mice were measured. The average of two swimming trials was used as an index of swimming endurance at each time point. The aged mice were assessed as exhausted when they failed to rise to the surface of water to breathe within a 7-s period; at this point they were immediately rescued from the tank (*13*). The swimming tests were performed between 11:00 and 17:00 to avoid circadian variations in physical activity; it has been demonstrated that minimal variation of endurance occurs in mice during this period of time (*13*).

Endurance training reportedly increases muscle endurance (*4*, *14*). Therefore, swimming training was carried out only once in this study for 10 min before each swimming test to avoid influencing the swimming times through increased training.

Sample collection and processing. At the end of the feeding trial and after 3 d for physical recovery from the fatigue of the swimming test, non-fasted aged mice were subjected to an additional swimming test for 5 min with a load of 1% of their body weights attached to their tails. The aged mice were then 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. Samples of liver and muscle tissue from the hind legs were then removed and stored at −30°C. A blood sample (0.2 mL) was immediately deproteinized with perchloric acid (0.6 M) and centrifuged at 3,000 ×*g* for 10 min for determination of plasma lactate. The residual blood sample was centrifuged at 900 ×*g* for 15 min, and the supernatant was stored at −30°C for determinations of plasma glucose and non-esterified fatty acid (NEFA) concentrations.

Biochemical analysis. The plasma glucose and NEFA were assayed by glucose oxidase and acyl-CoA oxidase methods using commercial kits (Glucose CII test Wako, and NEFA C-test Wako: Wako Pure Chemical Industries, Ltd., Osaka, Japan). The plasma and muscle L-(+)-lactates were measured using an enzymatic analysis with lactate dehydrogenase and NAD^+ (*15*). Liver and muscle glycogens were determined using an enzymatic method with amyloglucosidase (*16*).

Statistical analysis. The data were expressed as the means±SE. Comparisons of the swimming times to exhaustion of mice from the lard, fish oil, and sea snake lipid diet groups were assessed using two-way analysis

of variance (ANOVA), and biochemical parameters of mice among three diet groups were assessed using one-way ANOVA, and the Spjotvoll/Stoline test using the STATISTICA statistical program package (StatSoft Inc., Tulsa, OK, USA). A level of $p<0.05$ was used as the criterion for statistical significance.

RESULTS

Body weights and food intake

The body weights of the aged mice before the feeding trial were as follows: lard diet group, 47.5±0.7 g; fish oil diet group, 48.2±0.7 g; and sea snake lipid diet group, 47.6±0.8 g. The body weights of the aged mice after the feeding trial were as follows: lard diet group, 48.7±1.0 g; fish oil diet group, 47.0±1.1 g; and sea snake lipid diet group, 47.5±1.2 g. There were no significant differences in the body weights of the three diet groups before and after the feeding trial ($p>0.05$). There were no differences in the average dietary intake, which was 4.3 g/d for each group.

Swimming endurance

The swimming times to exhaustion before and after the feeding trials for the three diet groups are indicated in Fig. 1. The swimming times before the feeding trial among the three diet groups did not differ significantly ($p>0.05$). The swimming times to exhaustion of the sea snake lipid diet group tended to be longer after the feeding trial than those of the lard diet group, and were significantly extended compared with those of the fish oil diet group ($p<0.05$). The swimming times of the aged mice that were fed the fish oil diet were slightly lower than those of the lard diet group, but this difference was not statistically significant ($p>0.05$). Furthermore, in case of only time as a factor for the swimming endurance, the swimming times after the feeding trial as a whole were markedly lower than those before the feeding trial ($p<0.03$). However, for individual diet groups, the observed swimming times after the feeding trial were significantly lower than those before the feeding trial only in the fish oil diet group ($p<0.05$), whereas there was a tendency toward decreasing times in the lard diet group and increasing times in the sea snake lipid diet group.

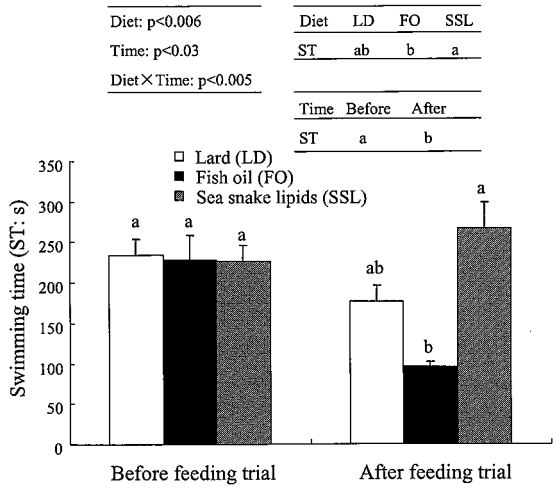

Fig. 1. Effects of lard, fish oil, and sea snake lipids on swimming times to exhaustion of aged mice before (each group: $n=17$) and after (lard: $n=14$; fish oil: $n=10$; sea snake lipids: $n=11$) feeding trials. Each value represents the mean±SE. Significant differences between the three diet groups before and after feeding trials are denoted by a, b, and ab ($p<0.05$).

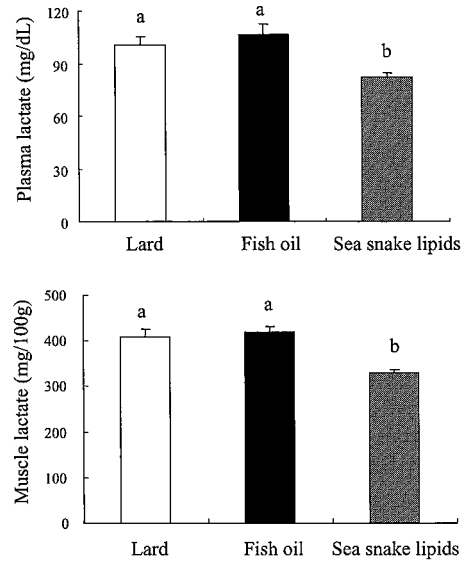

Fig. 2. Effects of lard, fish oil and sea snake lipids on lactate levels in plasma and muscles of aged mice supporting loads of 1% of their body weights while subjected to swimming for 5 min (lard: $n=14$; fish oil: $n=10$; sea snake lipids: $n=11$). Each value represents the mean±SE. Significant differences from the lard diet group are denoted by a and b ($p<0.05$).

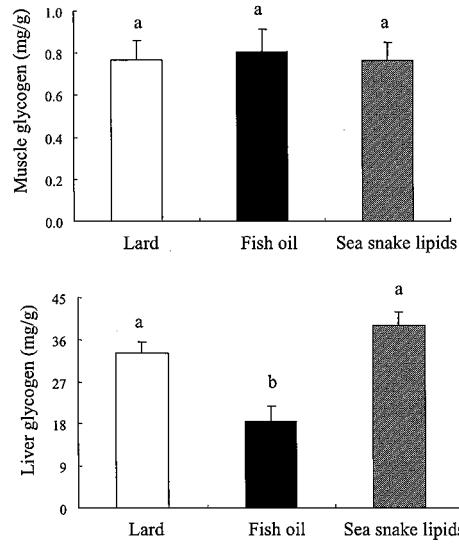

Fig. 3. Effects of lard, fish oil, and sea snake lipids on glycogen content in muscles and livers of aged mice supporting loads of 1% of their body weights while subjected to swimming for 5 min (lard: $n=14$; fish oil: $n=10$; sea snake lipids: $n=11$). Each value represents the mean±SE. Significant differences from the lard diet group are denoted by a and b ($p<0.05$).

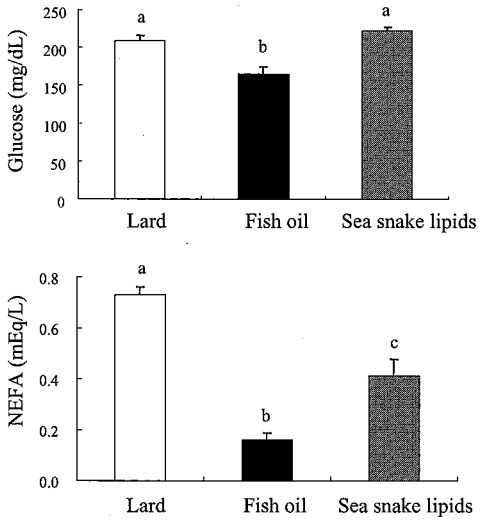

Fig. 4. Effects of lard, fish oil, and sea snake lipids on glucose and NEFA concentrations in plasma of aged mice supporting loads of 1% of their body weights while subjected to swimming for 5 min (lard: $n=14$; fish oil: $n=10$; sea snake lipids: $n=11$). Each value represents the mean±SE. Significant differences from the lard diet group are denoted by a, b, and c ($p<0.05$).

Biochemical parameters

The biochemical parameters were assessed in the plasma, skeletal muscles, and livers of the aged mice collected immediately after the final 5 min swimming exercise with a 1% load attached to their tails. The plasma and muscle lactate levels in the three diet groups are plotted in Fig. 2. Both the plasma and muscle lactate levels were significantly lower in the sea snake lipid diet group than in the lard or fish oil diet groups ($p<0.05$), but there were no significant differences between those levels in the fish oil and lard diet groups ($p>0.05$).

The muscle and liver glycogen levels in the three diet groups are indicated in Fig. 3. There were no marked differences in muscle glycogen levels among the aged mice from any of the three diet groups ($p>0.05$). The liver glycogen levels in the sea snake lipids and lard diet groups were significantly higher than those in the fish oil diet group ($p<0.05$), but there was no significant difference in these levels between the aged mice in the sea snake lipids and lard diet groups ($p>0.05$).

The plasma glucose and NEFA concentrations in the three diet groups are presented in Fig. 4. The plasma glucose concentrations of the sea snake lipids and lard diet groups were significantly higher than those of the fish oil diet group ($p<0.05$). Again there was no significant difference in plasma glucose between the sea snake lipids and lard diet groups ($p>0.05$). In contrast, the plasma NEFA concentrations in the sea snake lipid diet group were significantly lower than those of the lard diet group ($p<0.05$), but significantly higher than those of the fish oil diet group ($p<0.05$).

DISCUSSION

This study was designed to provide information regarding the swimming endurance of aged mice following a dietary intake of Erabu sea snake lipids. The swimming times to exhaustion before the feeding trial did not differ significantly among the three diet groups. The swimming times to exhaustion after the feeding trial revealed a marked improvement in the sea snake lipid diet group compared with those of the fish oil diet group. The levels of food intake and body weights were similar in all diet groups, suggesting that it was the sea snake lipid diet which improved the swimming endurance of aged mice rather than a secondary effect due to differences in buoyancy. In addition, the swimming times at Week 16 for the lard diet group revealed a decreasing tendency from the times at Week 0 that was not observed in young mice (*10*). The swimming times at Week 16 for the fish oil diet group were significantly lower; this decrease in endurance compared with the swimming times at Week 0 was not observed in the aged mice fed the sea snake lipid diet. These indicate that aging causes a decrease in endurance and that intake of sea snake lipids lessens that decrease and maintains the endurance of aged mice, and the intake of fish oil can accelerate this decreased endurance in aged mice.

Lactate is generated during anaerobic metabolism; its production in muscle is associated with fatigue (*17*). Lower lactate levels in both the plasma and muscle were observed in the sea snake lipid diet group than in the lard and fish oil diet groups following a 5 min swimming exercise, suggesting that those aged mice were less fatigued, which is consistent with their prolonged swimming times to exhaustion. Other researchers have reported that intake of medium-chain triglycerides (*18*), *Anoectochilus formosanus* (*19*), *Paecilomyces japonica* or *Grifola frondosa* extracts (*20*), dichloroacetate (*21*, *22*), red mold rice (*23*), or fresh royal jelly (*24*) significantly prolonged endurance, as indicated by lower lactate concentrations than in control groups. Intake of those components may attenuate production and/or enhance clearance of lactate during exercise. Sea snake lipid intake may have the same effect. Further research is necessary to ascertain if such a mechanism operates in mice with a dietary intake of sea snake lipids. It is of interest whether age affects this mechanism, since a similar decrease in lactate levels was observed in young mice fed sea snake lipids (*10*).

Glycogen deposits are important for maintaining physical performance during endurance exercises (*25*–*29*). We previously reported that young (12-wk-old) mice fed a sea snake lipid diet had significantly higher levels of muscle glycogen as well as plasma glucose levels than animals fed a lard diet following a 5 min swimming exercise; and so the improved swimming endurance is likely intimately related with the glycogen and plasma glucose metabolism as energies for swimming exercise in young mice (*10*). In contrast, there was no significant difference in the muscle glycogen and plasma glucose levels between the sea snake lipid and lard diet groups, suggesting that this relationship associated with the swimming endurance and glycogen metabolism, is not observed in aged mice. It has been

reported that the activities of some enzymes associated with the tricarboxylic acid (TCA) cycle, which is involved in the oxidation of glucose and glycogen and consequent energy production, are decreased during aging (*30–32*). It may be that less efficient energy production from glucose and glycogen in the aged mice compared with young mice during swimming exercise reduced any difference in glycogen utilization between the mice fed the sea snake lipids and lard diets. It was interesting that the aged mice fed the fish oil diet had markedly lower levels of plasma glucose and liver glycogen than those fed the lard diet. This may imply that there was a higher consumption of glycogen and glucose in the aged mice fed the fish oil diet than in those fed the lard diet, and so the intake of fish oil showed a decreased endurance as compared with those at Week 0 in aged mice. These results suggest that components of the various diets impact the regulation of glycogen and glucose oxidation; however, the mechanisms involved are complex and currently unknown.

Plasma NEFA is also a major fuel source for muscles during prolonged exercise (*33–35*). It has been reported that long-term intake of green tea extract in mice (*3*) or oral administration of capsaicin before exercise in rats (*36, 37*) enhances endurance. The plasma concentrations of NEFA in those studies were markedly higher in the test groups than in the control groups after 30 min of swimming exercise, suggesting an increase in fatty acid mobilization for utilization as an energy source. In our study, the plasma NEFA concentrations were significantly lower in the sea snake lipid diet group than in the lard group at a 5 min swimming exercise. This is discrepant with the tendency of swimming times for the two diet groups. This suggests that NEFA was perhaps not a major source of energy in the aged mice fed a sea snake lipid diet. It has been demonstrated that *n*-3 PUFAs, such as DHA, can lower plasma NEFA or plasma and liver lipids in animals (*8, 38*). The sea snake lipid and fish oil diets used in this study were rich in *n*-3 PUFAs (Table 1). The lower levels of plasma NEFA observed in both the sea snake lipids and fish oil diet groups compared with the lard diet group were probably due to the influence of *n*-3 PUFAs on lipid metabolism. This is supported by the observation that plasma NEFA levels in the mice that were fed the sea snake lipid diet were markedly higher than in those fed the fish oil diet, reflecting the relative levels of *n*-3 PUFAs in those two diets. However, it is also possible that the improved endurance observed in the sea snake lipid diet group resulted in a higher utilization of NEFA than in the fish oil diet group. Furthermore, the plasma NEFA concentrations of the aged mice fed the fish oil diet were significantly lower than those fed the lard diet. It suggests that the intake of fish oil decreased the endurance as compared to that at Week 0 for lower NEFA utilization. In addition, intake of a diet enriched with *n*-3 PUFAs did not improve endurance in other animal or human studies (*39, 40*). These various observations indicate that components of sea snake lipids other than *n*-3 PUFAs contributed to the swimming endurance of aged mice, but these factors and their mechanisms of action have yet to be identified.

In conclusion, long-term intake of Erabu sea snake lipids markedly improved the swimming endurance of healthy aged mice. This effect probably involves attenuation of lactate production and/or enhancement of lactate clearance. Conversely, the intake of fish oil which enriched *n*-3 PUFAs as did the sea snake lipids, decreased the endurance of aged mice. It is unlikely that *n*-3 PUFAs contained in the sea snake lipids make a major contribution to the improved endurance in aged mice. More research is required to clarify the complex mechanisms by which this improvement of endurance in aged mice occurs. The efficacy of sea snake lipids to improve endurance in humans has not been demonstrated; however, our results suggest that long-term dietary intake of sea snake lipids may be beneficial for maintaining the body strength of older individuals.

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