Original Communication

A Comparative Study of the Effects of Erabu Sea Snake (*Laticauda semifasciata*) Lipids, Green Tea Extract and Conjugated Linoleic Acid on the Swimming Endurance of Mice

Guihua Zhang[1], Nobuya Shirai[1], Tomoyuki Higuchi[2], Hiramitsu Suzuki[2], Eiji Shimizu[3]

[1] National Agriculture and Food Research Organization, National Food Research Institute, Tsukuba, Ibaraki, Japan
[2] Kagawa Nutrition University, Sakado, Saitama, Japan
[3] Fuji Pharmaceutical Inc., Meguro, Tokyo, Japan

Received for publication: March 11, 2009; Accepted for publication: August 3, 2009

Abstract: The aim of this study was to assess the interaction between physical and biochemical parameters in mice fed 1 % sea snake lipids (SSL) and compare these with animals fed diets containing 0.2 % green tea extract (GTE) or 0.5 % conjugated linoleic acid (CLA). The swimming times of the SSL group were significantly increased at Weeks 12 and 16 ($p<0.001$), and those of the GTE group, at Week 12 ($p<0.005$), but not those of the control or CLA group, compared with those at Week 0. The increase tended to be significant in the SSL group compared with the control group at Week 12 ($p=0.09$). Both the SSL and GTE groups had significantly longer swimming times than the CLA group at Weeks 12 and 16 ($p<0.001$). After 5 minutes of swimming exercise, the SSL group exhibited significantly lower levels of plasma and muscle lactates ($p<0.01$), and plasma non-esterified fatty acid (NEFA) ($p<0.001$) than the control group. There were no significant differences in any of plasma glucose, muscle and liver

 DOI 10.1024/0300-9831.79.5.362

glycogens, muscle lactate dehydrogenase (LDH), carnitine palmitoyltransferase (CPT), or monocarboxylate transporter 1 (MCT1) between SSL and control groups. The results suggest that the intake of 1 % SSL improved endurance more than the intake of 0.2 % GTE or 0.5 % CLA in mice. This action may involve the promotion of lactate oxidation for utilization.

Key words: sea snake lipids, green tea extract, conjugated linoleic acid, endurance, lactate, monocarboxylate transporter 1, mice

Introduction

Erabu sea snake (*Laticauda semifasciata*) is a marine reptile distributed throughout the Pacific Ocean and along the coast of the South China Sea. Lipids extracted from a fat sack (fat body) in the viscera of Erabu sea snake have been used as health-promoting food supplements in Japan [1]. In animal studies, an intake of sea snake lipids (SSL) can improve learning ability [2], and reduce plasma and liver lipids and plasma glucose levels in mice [3]. Our previous studies [4,5] have demonstrated that both young and aged mice, fed a 6 % SSL diet for 16 weeks, exhibited a marked improvement in swimming endurance, and this action was accompanied by decreased lactate accumulation and lower non-esterified fatty acid (NEFA) levels. Currently, the precise mechanisms by which these improvements were initiated have not been elucidated.

There are a number of sites at which the SSL might act. Glycogen and fatty acids are important energy substrates for exercise [6–9]. Glycogen sparing, e.g., promoting fatty acid oxidation, could prolong physical performance [10]. It has been shown that β-oxidation of fatty acid was promoted in mice by dietary supplementation with green tea extracts (GTE) for eight to ten weeks [11,12], or conjugated linoleic acid (CLA) for one week [13]. This increased fatty acid utilization was accompanied by significant improvements in the endurance of these mice. Whether such a mechanism also contributes to the improved endurance in the mice fed the SSL diet still needs to be clarified. SSL contain mostly long-chain fatty acids (Table I). These long-chain fatty acids may be ingested by mice and oxidized for energy production during swimming exercise, and this action might be connected with the activity of carnitine palmitoyltransferase (CPT), which is an important transporter of long-chain fatty acids across the mitochondrial membrane for oxidative metabolism [14,15]. Furthermore, it has been reported that skeletal muscle can both produce lactate and metabolize lactate for energy production [16,17] following its transport across the sarcolemmal membrane by a member of the monocarboxylate transporter (MCT) family, in which the expression of MCT1 was highly correlated with lactate utilization [18–20]. Whether decreased lactate accumulation observed in the SSL-ingesting mice during swimming exercise [4,5] is associated with the MCT1 protein level is still to be determined.

In addition, lipid peroxidation, which can be readily monitored by levels of thiobarbituric acid-reactive substances (TBARS), is a major indicator of oxidative stress, which may reflect a certain state of fatigue during swimming exercise [21]. Moriura *et al.* [22] have indicated that prolonged swimming times in the rats administered 50 % ethanol extract from the dried whole body of *Agkistrodon blomhoffii blomhoffii* BOIE, which is a venomous snake found throughout Japan, was attributed to the inhibition of TBARS elevation during swimming exercise. It is conceivable that intake of SSL may reduce the extent of lipid peroxidation.

The aim of this study is to explore the mechanisms of endurance improvement in the mice following the administration of SSL. Therefore, we compared the effects of 1 % SSL with 0.2 % GTE and 0.5 % CLA, both of which have been known to improve endurance through promoting fatty acid oxidation, on the physical and biochemical parameters in the mice.

Materials and methods

Animals

Male Crlj:CD-1 (ICR) mice aged 4 weeks were purchased from Charles River Japan Inc. (Atsugi, Kanagawa, Japan), and housed in cages (10 mice per cage) under automatically controlled conditions of temperature (24±0.5°C), relative humidity (65±5 %), and lighting (light from 06:45 to 18:45) for 18 weeks. Forty-eight mice were randomly divided into four groups (12 in each group; one cage was divided into two sections by a steel plate, and each section contained 4 mice), with equal body weights

and swimming times to exhaustion at 22 weeks of age. They were then switched from laboratory chow MF (Oriental Yeast Co., Ltd., Tokyo, Japan) to four different experimental diets (control, GTE, CLA, and SSL diets). Animals had free access to the experimental diets and water for 16 weeks when they were sacrificed. Body weights of mice were determined every 2 weeks. All animal procedures were reviewed and approved by the Animal Research Committee of the National Food Research Institute, National Agriculture and Food Research Organization (NARO), Japan.

Table I: Compositions of the experimental diets (%)

	Dietary group			
	Control	GTE	CLA	SSL
Corn starch	47.8	47.6	47.8	47.8
Casein	20	20	20	20
Granulated sugar	15	15	15	15
Cellulose	5	5	5	5
Mineral mixture	4	4	4	4
Vitamin mixture	2	2	2	2
L-methionine	0.2	0.2	0.2	0.2
Lard	6	6	5.5	5
Green tea extract	-	0.2	-	-
Conjugated linoleic acid	-	-	0.5	-
Sea snake lipids	-	-	-	1

GTE: green tea extract; CLA: conjugated linoleic acid; SSL: sea snake lipids. Lard contains 16:0 (25.3 %), 18:0 (12.5 %), 16:1 (3.4 %), 18:1n-9 (41.2 %), 18:1n-7 (2.9 %), and 18:2n-6 (8 %); Green tea extract contains more than 80 % catechin; Conjugated lineolic acid contains 82 % cis-9, trans-11 and trans-10, cis-12, and the others are 10 % 18:1n-9, 5 % 16:0, 2 % 18:0, and 1 % 18:2n-6; Sea snake lipids contains 22:6n-3 (12 %), 20:5n-3 (1.5 %), and the others are 16:0 (30 %), 16:1 (5.7 %), 18:0 (8.5 %), 18:1n-9 (22.4 %), 18:1n-7 (2.3 %), 18:2n-6 (1.4 %), 20:4n-6 (1.6 %)

Experimental diets

The lard was supplied by NOF Co., Ltd. (Tokyo, Japan), and Erabu SSL, by Fuji Pharmaceutical Inc. (Tokyo, Japan). Lard primarily contained 16:0 (25.3 %), 18:0 (12.5 %), 16:1 (3.4 %), 18:1n-9 (41.2 %), 18:1n-7 (2.9 %), and 18:2n-6 (8 %) fatty acids. SSL are rich in n-3 polyunsaturated fatty acids (PUFAs): e.g., 22:6n-3 (12.0 %) and 20:5n-3 (1.5 %). Other SSL fatty acids are primarily 16:0 (30 %), 16:1 (5.7 %), 18:0 (8.5 %), 18:1n-9 (22.4 %), 18:1n-7 (2.3 %), 18:2n-6 (1.4 %), and 20:4n-6 (1.6 %). GTE, Polyphenon 70 S, was purchased from Mitsui Norin Co., Ltd. (Tokyo, Japan) and contained more than 80 % catechin. CLA, extracted from safflower seed, was obtained in capsule form (BeeBest CLA 500) from BeeBest Co. Ltd. (Tokyo, Japan). The conjugated linoleic acid contained 82 % cis-9, trans-11 and trans-10, cis-12 isomers; the remaining fatty acids are 10 % 18:1n-9, 5 % 16:0, 2 % 18:0, and 1 % 18:2n-6. The content (liquid part) of CLA was used and mixed into the diet. The compositions of the four experimental diets (control, GTE, CLA, and SSL diets) are given in Table I. Mineral and vitamin mixtures were obtained from Oriental Yeast Co., Ltd. The amounts of GTE (catechin, 0.2 %), CLA (0.5 %), and SSL (1 %) added to the diets were calculated according to the range of tolerable daily intake of humans. Each experimental diet was stored below -40°C to prevent oxidative changes in fatty acid composition during storage.

Swimming endurance

The forced swimming test has been described in our previous studies [4,5]. Briefly, a soapless soap solution (1 % neutral kitchen detergent, Kao Co., Ltd., Japan) was used to wash the bodies and thoroughly moisten the hairs of the mice before the swimming exercise, to prevent any buoyancy differences caused by hairs [23,24]. Swimming times to exhaustion of mice supporting 2 % loads corresponding to the body weights attached to their tails were determined in a tank (26×22×29 cm) filled with water to a depth of 25 cm at a temperature of 23°C, and were used as indices of swimming endurance. Mice were assessed as exhausted when they failed to rise to the surface of the water to breathe within a 7-second period [25]. The swimming tests were carried out every 4 weeks between 11:00 and 17:00 hours to avoid circadian variations in physical activity [25]. On the day prior to each test, mice received swimming training for 10 minutes with no loads attached to their tails in preparation for the swimming exercise. This was only performed once in order to avoid any possibility of influencing swimming times by increased swimming training, which had been demonstrated by some studies [26,27].

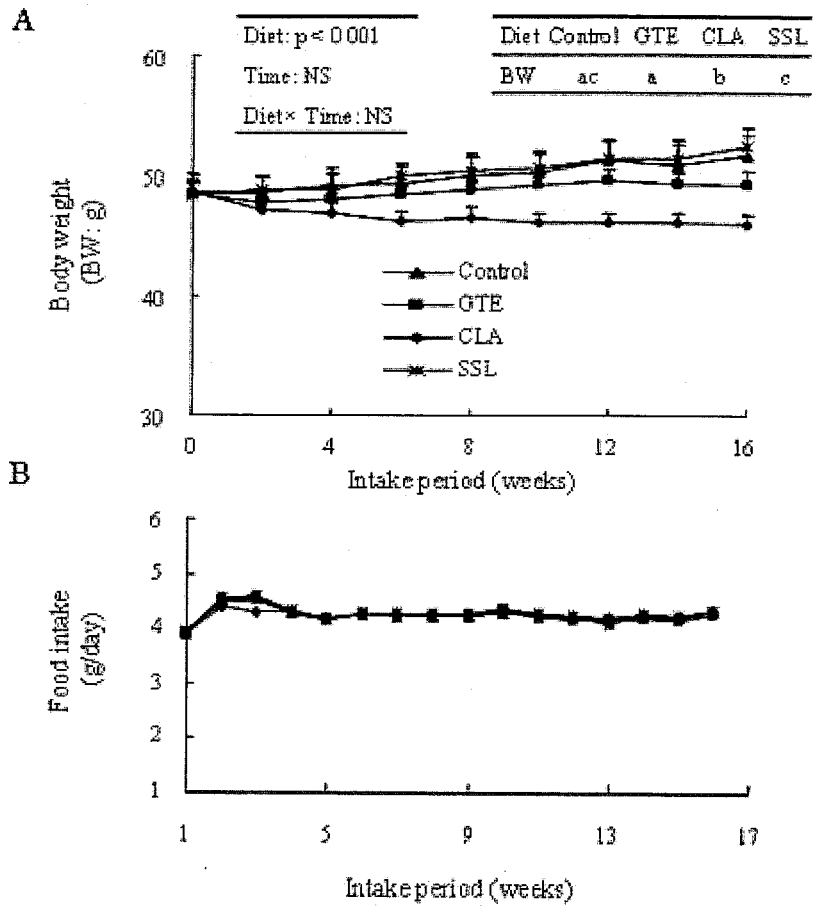

Figure 1: Time course of changes in body weight (A) and food intake (B) in mice fed the control diet, and diets supplemented with 0.2 % green tea extract (GTE), 0.5 % conjugated linoleic acid (CLA), or 1 % sea snake lipids (SSL) (n=12 for each). Significant differences in the body weight among the four diet groups are denoted by a, b, ab, and c ($p<0.05$, NS= non-significant).

Sample collection and processing

At the end of 16 weeks of feeding and after 3 days of physical recovery from the first swimming test, non-fasted mice were subjected to a further swimming test for 5 minutes with loads that were 1 % of body weight and immediately sacrificed by dislocation of the neck. Blood samples were collected from the caudal vena cava with a heparinized syringe and put into ice-cold tubes. Samples of liver and hind limb muscle of mice were immediately removed and rapidly frozen using liquid nitrogen. Liver for estimating glycogen and muscle tissue for assessing lactate, glycogen, CPT, lactate dehydrogenase (LDH), and MCT1 were stored at -60°C. A blood sample (0.2 mL) was deproteinized with perchloric acid (0.6 M) and centrifuged at 3,000 × g for 10 minutes to determine plasma lactate. The remaining blood sample was centrifuged at 900 × g for 15 minutes, and the supernatant was stored at -40°C for determining plasma glucose, NEFA, and TBARS concentrations. Frozen muscle samples were homogenized in buffer (0.25 M sucrose, 1 mM EDTA, and 3 mM tris-HCl, pH 7.2) and were centrifuged at 500 × g for 10 minutes. Half of the resulting supernatant was used for determining CPT activity, and the other half was again centrifuged at 10,000 × g for 10 minutes to yield a supernatant for measuring LDH activity. For glycogen determination, frozen samples of muscle and liver were homogenized with 0.6M perchloric acid, and homogenates were stored at -60°C for analysis.

Determining biochemical parameters

Plasma glucose and NEFA concentrations were measured using commercial kits (Glucose CII-test Wako, NEFA C-test Wako, Wako Pure Chemical Industries, Osaka, Japan). Plasma TBARS was measured by the method of Yagi [28]. Plasma and muscle L-(+)-lactate [29] and muscle and liver glycogens [30] were measured using the published enzymatic methods. CPT activity in muscle was determined using palmitoyl-CoA as the substrate [31], and LDH activity, using pyruvate and NADH as substrates

[32]. CPT and LDH activities were adjusted to the total supernatant protein determined by the Lowry method [33].

MCT1

Frozen muscle tissue (100 mg) was homogenized in 2 mL of buffer A [210 mM sucrose, 2 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 40 mM NaCl, 30 mM N-2-hydroxy-ethylpiperazine-N'-2- ethanesulfonic acid, 5 mM EDTA, 2 mM phenylmethyl-sulfonyl fluoride, pH 7.4] with two 20-second bursts with a Physcotron homogenizer set at 40 with a thick generator shaft (NS-10). Homogenates were transferred to centrifuge tubes, and 2 mL of buffer A used to rinse the homogenizer was added to the tubes. Three milliliters of buffer B (1.167 M KCl, 58.3 mM tetrasodium pyrophosphate) was then added. The homogenates were then mixed briefly and placed on ice for 15 minutes. After centrifugation at 230,000 × g for 75 minutes at 4°C, the supernatant was discarded and the pellet was washed thoroughly with 2 mL of buffer C (10 mM Tris (hydroxymethyl) aminomethane (Tris) base, 1 mM EDTA, pH 7.4). The pellet was resuspended in 600 µL of buffer C and homogenized by two 15-second bursts with a Physcotron homogenizer set at 40 with a thin generator shaft (NS-4). Two hundred microliters of 16 % sodium dodecyl sulfate was added to the samples, which were then mixed and centrifuged at 1,100 × g for 20 minutes at room temperature. The supernatant was divided into aliquots for measuring total protein content and MCT1 protein level and stored at -60°C.

To detect MCT1 protein, the supernatants were mixed with buffer D (10 M urea, 100 mM Tris-HCl, 2 % SDS, 0.08 % bromophenol blue) in a 3:1 (v/v) ratio. Protein samples (protein content: 75 µg) were separated on 9.6 % polyacrylamide gels (stacking gel: 3 %). The gels were then incubated in buffer E (0.1 M Tris-HCl, 0.192 M glycine, 20 % methanol) for 30 minutes prior to transfer to the polyvinylidene difluoride (PVDF) membrane (pre-incubated in buffer E) at 150 mA for 90 minutes. The membrane was incubated on a shaker overnight in buffer F [1 mM Tris-HCl, pH 8.0, 7.5 mM NaCl, 0.1 % (v/v) Tween 20, and 5 % (w/v) nonfat dried milk] containing goat anti-mouse antibody for MCT1 protein (1:500, Santa Cruz Biotechnology, Inc.) at 4°C. The membrane was washed in buffer F without dried milk and incubated for 1 hour with the rabbit anti-goat secondary antibody for MCT1 protein (1:4000, Chemicon) in the same buffer. MCT1 protein was detected using an alkaline phosphatase conjugate substrate kit (Bio-RAD). The MCT1 protein was quantified by scanning the PVDF membrane and analyzing band optical density with Scion Image (free software).

Statistical analyses

Data were expressed as means ± SEM. Body weights and swimming times of the control, GTE, CLA, and SSL groups were compared using a two-way analysis of variance (ANOVA). Comparisons of biochemical parameters and MCT1 protein levels were assessed using a 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 weight and food intake

The changes in body weight and the food intake of mice fed the control, GTE, CLA, and SSL diets over 16 weeks are presented in Figure 1. There were no significant differences in the body weights in any of these four groups of mice at any point of the feeding trial (Figure 1A). Body weights of the SSL, GTE, and control groups tended to increase with 16 weeks of feeding, unlike the CLA group for which body weight declined over time. Overall, the SSL group exhibited the greatest body weight increase, and the GTE group exhibited less increase than control group. There were no differences in the average dietary intake (Figure 1B), which was 4.3 g/day for each group.

Swimming endurance

The swimming endurances of mice on the four diets with loads corresponding to 2 % of their body weights are shown in Figure 2. Overall, the mean swimming times of mice fed the SSL diet were longer at all time points than those of the other groups, but these differences from the control or GTE group did not achieve statistical significance. The slight increases in swimming times of the SSL diet group from those in the control group observed at Weeks 4 and 8 were a bit more pronounced at Weeks 12 ($p=0.09$) and 16 ($p=0.13$). At Week 16, the swimming times for the

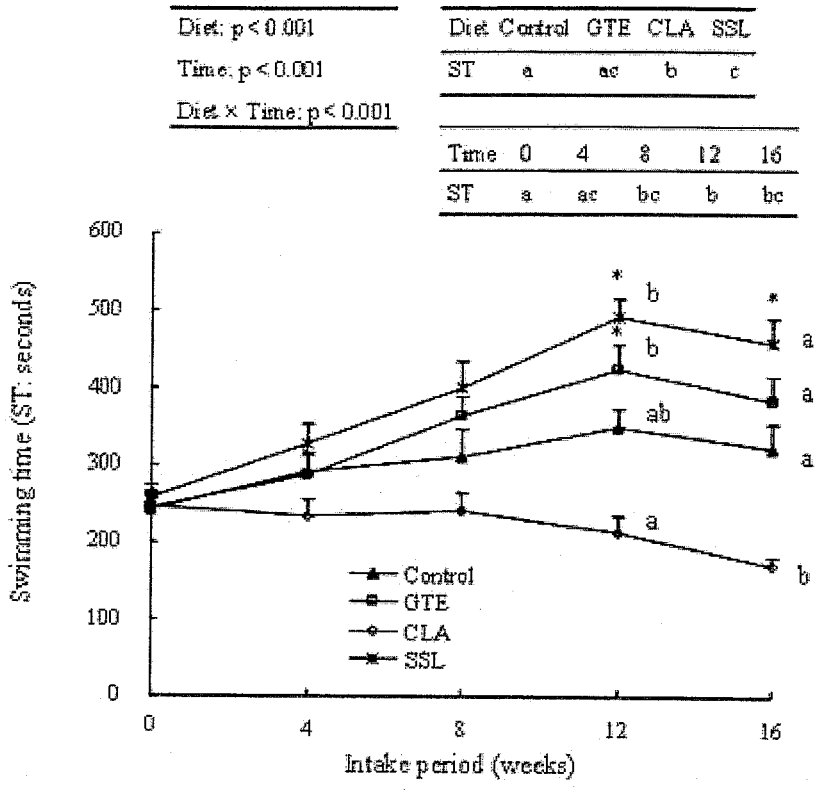

Figure 2: Effects of diets supplemented with 0.2 % green tea extract (GTE), 0.5 % conjugated linoleic acid (CLA), and 1 % sea snake lipids (SSL) on the swimming times of mice attached with constant loads corresponding to 2 % of their body weights (n=12 for each). Significant differences among four diet groups or intake periods are denoted by a, b, and c ($p<0.05$); CLA versus control: $p=0.08$ at Week 12; SSL versus control: $p=0.09$ at Week 12, and $p=0.13$ at Week 16. Significant differences from the swimming time of Week 0 in each diet group by * ($p<0.05$); GTE group, Week 16: $p = 0.13$.

SSL, GTE, and control groups were significantly longer than for the CLA group ($p<0.001$, $p<0.001$, and $p<0.05$). At Week 12, the swimming times for the CLA group were significantly shorter than those for the GTE and SSL groups ($p<0.001$ for both), and slightly shorter than those of the control group ($p=0.08$).

For the SSL and GTE diet groups, the mean swimming times from Week 8 onwards were longer than those at Week 0. The swimming times of the SSL group at Weeks 12 and 16 were significantly increased compared with those at Week 0 ($p<0.001$ for both). In the GTE group, the swimming times at Week 12 were significantly longer than at Week 0 ($p<0.005$), but this was not maintained at Week 16 ($p=0.13$). Swimming times in the control group tended to increase over time, while those in the CLA group tended to decrease. These results suggest that intake of 1 % SSL improves endurance more than intakes of 0.2 % GTE or 0.5 % CLA. In addition, long-term intake of CLA failed to improve endurance in mice, which is not consistent with the study of Mizunoya *et al.* [13].

Glycogen metabolism

The muscle and liver glycogen content and plasma glucose concentrations after 5 minutes of swimming in the mice fed the four experimental diets are presented in Figure 3. Muscle glycogen levels (Figure 3A) after swimming were similar in the SSL, GTE, and control groups. Mice in the CLA group had significantly lower levels of muscle glycogen than those in the control and GTE groups ($p<0.05$ for both), and the SSL group ($p<0.01$). Liver glycogen (Figure 3B) and plasma glucose (Figure 3C) levels following swimming were not markedly different among the four diet groups.

Lactate metabolism

The lactate content in plasma and muscle, and LDH activities in muscle after 5 minutes of swimming in the four experiment groups are presented in Figure 4. Plasma (Figure 4A) and muscle (Figure 4B) lactate levels of mice in the SSL group were significantly lower than those of animals in the control ($p<0.01$) or CLA ($p<0.001$) group. The differences in lactate levels between the SSL and GTE groups approached significance (plasma, $p=0.06$; muscle, $p=0.08$). Lev-

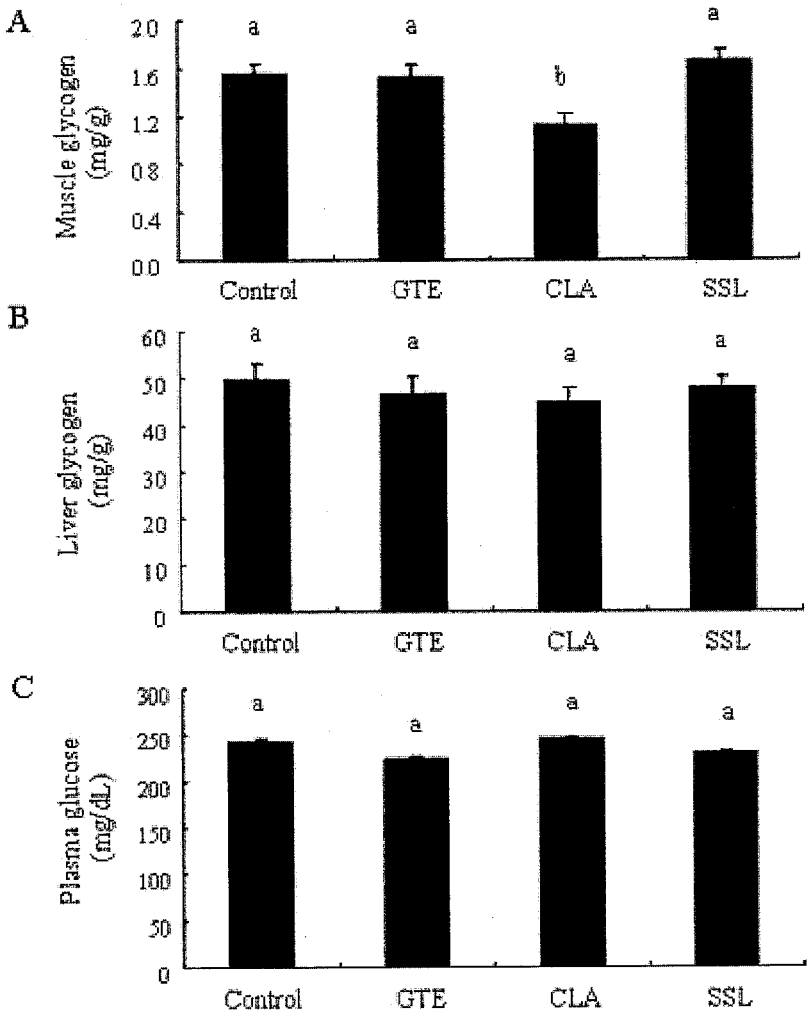

Figure 3: Effects of 0.2 % green tea extract (GTE), 0.5 % conjugated linoleic acid (CLA), and 1 % sea snake lipids (SSL) on muscle (A) and liver (B) glycogen levels and plasma glucose concentration (C) in mice with attached constant loads corresponding to 1 % of their body weights after 5 minutes of swimming (n=12 for each). Significant differences among the four diet groups are denoted by a, b ($p<0.05$).

els of lactate in the CLA group were statistically significantly higher than those in the control ($p<0.05$), GTE ($p<0.005$), and SSL ($p<0.001$) groups. The lactate levels from plasma and muscle in the control and GTE groups were similar. LDH levels in muscle were not markedly different among the four groups (Figure 4C).

The MCT1 protein levels of muscle after 5 minutes of swimming in the four experimental groups are shown in Figure 5. There were no significant differences in the MCT1 protein levels among the four experimental groups. Although the level of MCT1 appeared to be higher in the CLA group compared with the other groups, this difference was not statistically significant.

Lipid metabolism

Plasma NEFA and TBARS levels and muscular CPT activities in the four experimental groups after 5 minutes of swimming are depicted in Figure 6. The NEFA concentrations (Figure 6A) of the SSL group were significantly lower than those of the control and GTE groups ($p<0.001$ for both). Those in the CLA group were slightly lower than in the SSL group ($p=0.07$), and significantly lower than those in the control and GTE groups ($p<0.001$ for both). The GTE and control groups had similar NEFA concentrations (Figure 6A). The CPT activities in muscle were not significantly different between any of the four groups (Figure 6B).

The plasma TBARS concentrations decreased in all groups compared with the control group, but this was only significant in the GTE and CLA groups ($p<0.001$ for both), and not in the SSL group

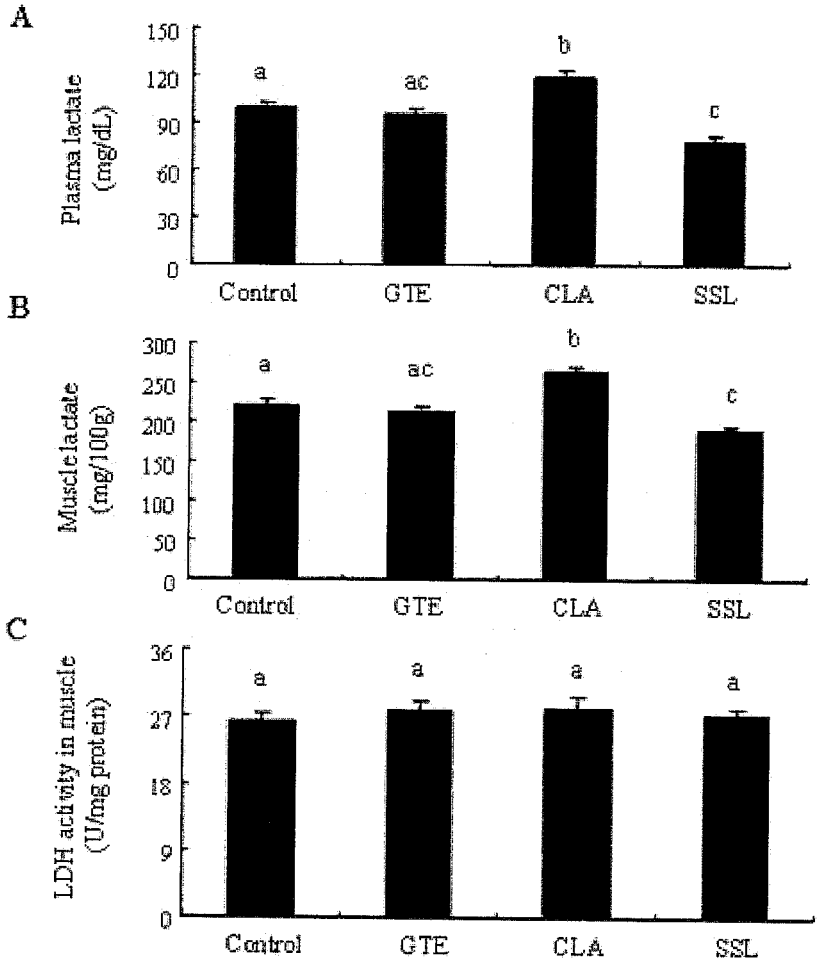

Figure 4: Effects of 0.2 % green tea extract (GTE), 0.5 % conjugated linoleic acid (CLA), and 1 % sea snake lipids (SSL) on plasma (A) and muscle (B) lactates, and muscle lactate dehydrogenase (LDH) activity (C) in mice with attached constant loads corresponding to 1 % of their body weights after 5 minutes of swimming (n=12 for each). Significant differences among the four diet groups are denoted by a, b, and c ($p<0.05$); SSL versus GTE: $p=0.06$ for plasma lactate, $p=0.08$ for muscle lactate.

($p=0.10$). The decrease was not significantly different between the SSL or CLA group and the GTE group ($p=0.17$, $p=0.77$). The decrease observed in the SSL group was significantly less than that observed in the CLA group ($p<0.05$) (Figure 6C).

Discussion

The objective of this study was to assess physical and biochemical parameters in mice fed a supplement of SSL and compare these with animals fed a control diet or diets supplemented with GTE or CLA. It is commonly accepted that swimming is a good experimental exercise model, and the biochemical parameters assessed previously in various studies have been associated with increased exercise endurance. The review of Larsen *et al.* [34] evaluated the safety of CLA supplementation and implied the toxicity of CLA. However, in the present study, the mice in the CLA group did not die during long-term intake, and the food intake was the same as in the other diet groups, suggesting that 0.5 % CLA may be safe in mice.

In terms of physical endurance, all experimental groups except the CLA group exhibited improved swimming times compared to the control group. The SSL group achieved the greatest improvement in endurance, and this was also the group that exhibited the greatest increase in body weight. In contrast, only the CLA group, for which the body weight declined over time, exhibited decreased endurance. It appeared that there was some correlation between swimming endurance and body weight change. In a previous study of mice with an intake of 0.5 % CLA over one week, swimming endurance was enhanced with the same levels of body weight for the CLA and control groups [13]. This observation is inconsistent with the result of the present study. It has been indicated that long-term intake of CLA decreased body fat and resulted in lower body weight in experimental animals [35–39]. These observations

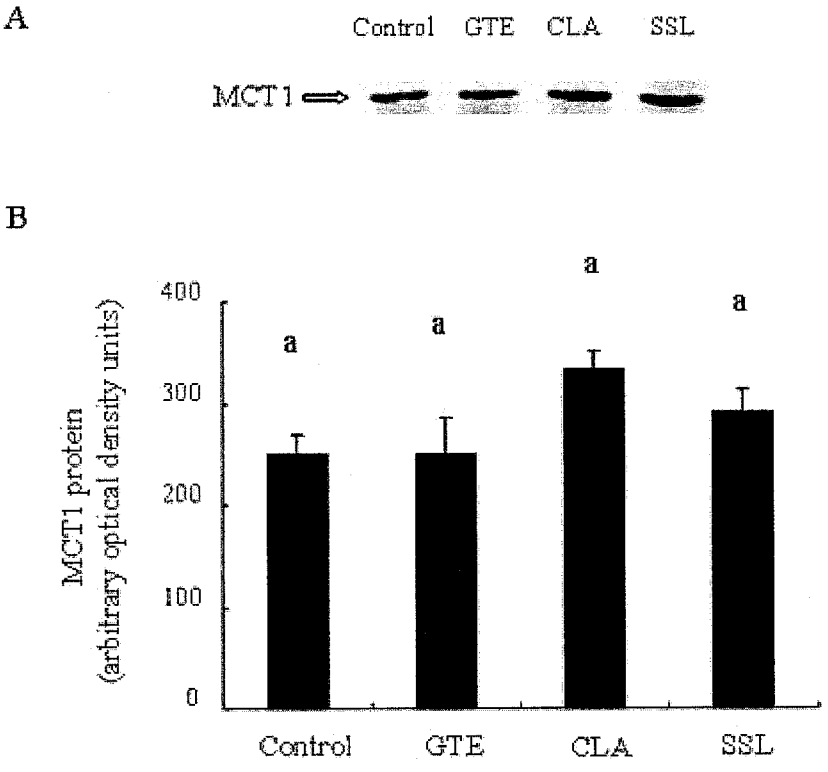

Figure 5: Representative immunoblots of the monocarboxylate transporter (MCT)1 in skeletal muscle samples from the control, 0.2 % green tea extract (GTE), 0.5 % conjugated linoleic acid (CLA), and 1 % sea snake lipids (SSL) diet groups (A), and their quantification (B) in mice (n=12 for each) with attached constant loads corresponding to 1 % of their body weights after 5 minutes of swimming. Significant differences among the four diet groups are denoted by a, b ($p<0.05$). The quantification of MCT1 protein was performed by scanning the PVDF membrane and analyzing band optical density with Scion Image (free software).

suggest that decreased body weight observed in the mice fed the CLA diet is attributed to reduced body fat, and this decreased body fat may decrease endurance perhaps due to increased energy expenditure caused by lower buoyancy, although relatively lower loads (2 % of the body weight of mice) were attached to the tails of mice fed the CLA diet during swimming exercise. Despite these associations between endurance and body weight change, the GTE group, which had less body weight increase than the control group, had greater endurance. This lesser body weight increase in the GTE group relative to the control group may be due to decreased body fat, which has been demonstrated in other studies [40,41]. These observations suggest that additional mechanisms of enhancing physical endurance must operate following an intake of various dietary supplements.

In the present study, we determined the biochemical parameters after 5 minutes of swimming exercise and investigated the differences of the energy substrates at this time point of exercise. From these data, we could estimate the available energy for use from 5 minutes to exhaustion. The CLA group had a significantly lower level of muscle glycogen but not liver glycogen or plasma glucose, suggesting that lower muscle glycogen content may contribute to decreased endurance. However, muscle glycogen and plasma glucose levels of mice fed the SSL diet were similar to those of the control group even though the swimming endurance of the former group was greater. This suggests that improved endurance was not directly dependent on glycogen metabolism as an energy source in the SSL group. In our previous study of mice with a higher intake of SSL (6 % versus 1 % in the present study), muscle glycogen levels after 5 minutes of swimming exercise and swimming endurance were both greater than in the control group [4]. Thus, the regulation of glycogen metabolism may differ depending on the level of SSL intake. Together, these observations suggest that various diets may produce different regulation of glycogen metabolism; further study is needed to clarify this.

The levels of plasma and muscle lactates after 5 minutes of swimming exercise were correlated with swimming endurance in the present study. The SSL diet group with the lowest lactate level had the longest swimming time, whereas the CLA group with the highest lactate level had the shortest swimming time. The lower lactate level of both plasma and muscle observed in the SSL group compared with the control group were consistent with the results of our previous studies [4,5]. These observations, i.e. that decreased lactate accumulation may be associated with increased endurance, have also been supported

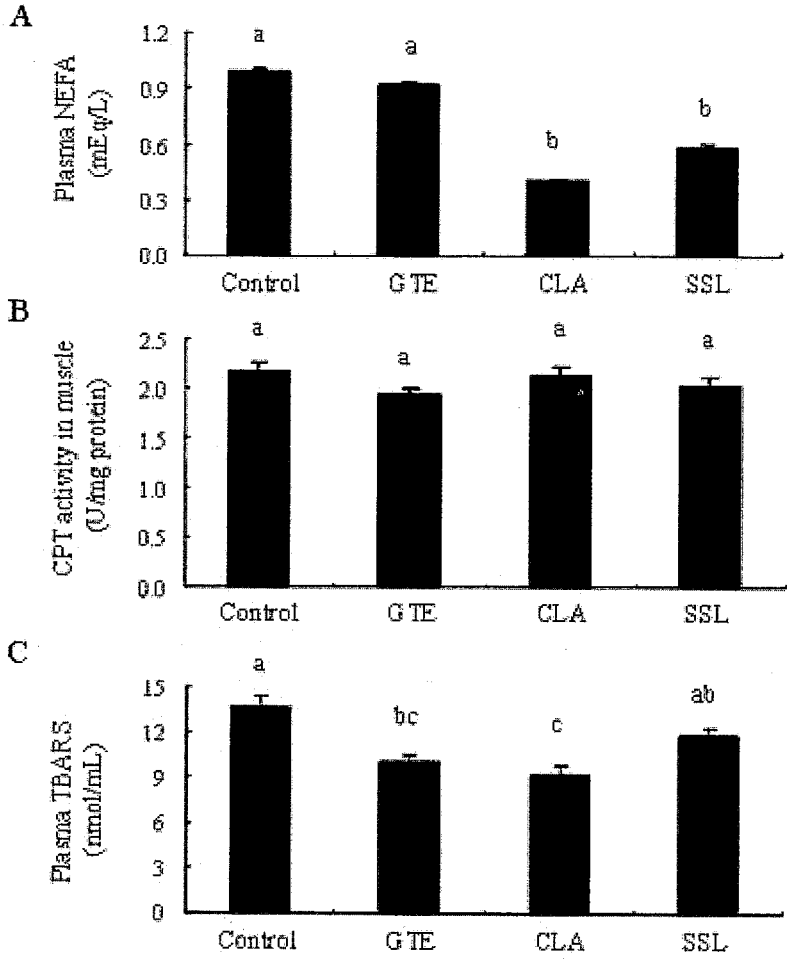

Figure 6: Effects of 0.2 % green tea extract (GTE), 0.5 % conjugated linoleic acid (CLA), and 1 % sea snake lipids (SSL) on plasma non-esterified fatty acids (NEFA, A), muscle carnitine palmitoyl-transferase (CPT) activity (B) and plasma thiobarbituric acid-reactive substances (TBARS, C) levels in mice with attached constant loads corresponding to 1 % of their body weights after 5 minutes of swimming (n=12 for each). Significant differences among the four diet groups are denoted by a, b, and c ($p<0.05$); SSL versus CLA: $p=0.07$ for plasma NEFA; SSL versus control or GTE: $p=0.10$, $p=0.17$ for plasma TBARS respectively.

by other studies [42–44]. A decrease in lactate accumulation may imply less glycolytic and more oxidative metabolism. However, in the present study, the activities of LDH that catalyze the reversible conversion of pyruvate to lactate were similar between the SSL and control groups after 5 minutes of swimming exercise. This result could indicate that there is no difference in glycolytic flux regarding lactate production between these two groups. Moreover, lactate produced in muscle during exercise can be metabolized following its transport across the sarcolemmal membrane [16,17]. It is possible that the lower lactate levels in the SSL group are a consequence of faster lactate utilization by oxidative metabolism. The transport of lactate across sarcolemmal membrane associated with MCT1 and the expression of the gene for this protein were highly correlated with lactate oxidation in rat muscle [18–20]. However, the MCT1 levels in skeletal muscle after 5 minutes of swimming exercise were similar between the SSL and control groups in this study. If the reason for the lower lactate levels in the SSL group is faster lactate utilization, then the mechanisms involved are probably associated with a post-transmembrane pathway, and further studies are necessary to determine such mechanisms.

NEFA is are also an important energy source for exercise, and the mobilization and utilization of NEFA is could enhance exercise performance [10–12,45]. Some studies [35–39] have indicated that intake of CLA decreases body fat in mice. This would have resulted in lower NEFA levels prior to swimming exercise in the mice fed CLA [13]. Again, significantly lower plasma NEFA and similar CPT activities after 5 minutes of swimming exercise were observed between the CLA and control groups in this study. These results suggest that decreased NEFA for utilization attenuated the swimming time. Similarly, intake of GTE could decrease fat by increased fat oxidation in experimental animals [40,41], ultimately lowering NEFA levels before swimming exercise in the GTE group [46]. Further, after 5 minutes of

swimming exercise, the same levels of NEFA between GTE and control groups were observed in the present study, even though the activity of CPT was not significantly different between these two groups. These results imply that the higher levels of NEFA may have been caused during swimming exercise in the mice following GTE and therefore contributing to the higher endurance by increasing NEFA metabolism to provide energy. In addition, the significantly lower NEFA levels observed in the SSL group compared with the control group may be due to the suppression of fatty acid synthesis by n-3 PUFAs contained in SSL [47,48]. It has also been reported that the rats fed a diet supplemented with n-3 fatty acids had a higher respiratory quotient than those fed a lard diet as control, indicating more carbohydrate and less fat oxidation for energy production [49]. Such a mechanism may also have applied to the mice fed the SSL-enriched n-3 PUFAs in this study. From these observations, it would seem that the mechanism of improved swimming endurance following intake of SSL is unlikely to involve enhanced fatty acid oxidation during swimming exercise.

Results of a study in which rats were fed an extract from the dried body of "Mamushi" indicated that its anti-fatigue effect during swimming exercise was associated with decreased TBARS levels [22]. In this study, the GTE group had lower plasma TBARS levels than the control group, and this was consistent with other studies [50,51] in which green tea extract ameliorated oxidative stress. Although plasma TBARS levels of the SSL group, which had the greatest swimming endurance, tended to decrease compared with the control group ($p=0.10$), the CLA group, which had the lowest swimming time, had significantly lower TBARS levels than both the control and SSL groups. Therefore, it is very unlikely that the improved endurance observed in the SSL group is primarily due to inhibition of lipid peroxidation during swimming exercise.

In summary, long-term intake of a diet supplemented with 1 % Erabu SSL was more beneficial in improving swimming endurance than diets containing 0.2 % GTE and 0.5 % CLA. The evidence suggests that promotion of lactate oxidation for utilization may contribute to this effect, but fatty acid mobilization and utilization did not appear to be involved in improved swimming endurance. Certainly, we also could not exclude other mechanisms being involved; e.g., hormone regulation. Further studies are needed to clarify the complex mechanisms associated with the improved swimming endurance of mice fed SSL. From our observations, it would be expected that a dietary intake of Erabu SSL is beneficial in improving physical performance in humans.

Acknowledgements

This study was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries (MAFF) for the food research project.

Abbreviations

ANOVA, analysis of variance; BW, body weight; CLA, conjugated linoleic acid; CPT, carnitine palmitoyltransferase; GTE, green tea extract; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; NARO, National Agriculture and Food Research Organization; NEFA, non-esterified fatty acid; NS, non-significant; PUFAs, polyunsaturated fatty acids; PVDF, polyvinylidene difluoride; SSL, sea snake lipids; ST, swimming time; TBARS, thiobarbituric acid-reactive substances

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Nobuya Shirai

National Agriculture and Food Research Organization
National Food Research Institute
2-1-12, Kannondai, Tsukuba
Ibaraki 305–8642, Japan
Tel: +81 29 838 8083, Fax: +81 29 838 7996
E-mail: nshinya@affrc.go.jp