

1 **Effect of dietary glucosylceramide from sea cucumber on plasma and liver lipids in**  
2 **cholesterol-fed mice**

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18

1 **Abstract**

2  
3 Various physiological functions of dietary glucosylceramides (GlcCer), such as  
4 preventing colon cancer and improving the skin barrier function, have been reported.  
5 One of the potential GlcCer sources used as a foodstuff is sea cucumber. In this study,  
6 our objective was to determine the effect of dietary GlcCer prepared from sea cucumber  
7 on plasma and liver lipids in cholesterol-fed mice. ICR mice were fed four different diets  
8 (control diet, sea cucumber GlcCer supplemented diet, high cholesterol supplemented  
9 diet and high cholesterol + sea cucumber GlcCer supplemented diet). Dietary GlcCer  
10 decreased total cholesterol significantly in ICR mice. The mRNA expression of LDL  
11 receptor was increased significantly while the gene CYP7A1 involved in bile acids  
12 formation was decreased significantly comparing with control (diet without cholesterol).  
13 These results suggested that modulation of cholesterol homeostasis gene in liver was  
14 due to cholesterol lowering effect of dietary GlcCer.

15  
16 **Keywords** glycosylceramide, sphingolipids, sea cucumber, cholesterol, lipid  
17 metabolism, mice.

18

## 1 **Introduction**

2       The role of functional foods in preventing various chronic diseases (e.g.  
3 cardiovascular disease, allergies, cancer) has been focused increasingly. Sphingolipids  
4 are highly bioactive compounds that participate in the regulation of cell growth,  
5 differentiation, diverse cell functions, and apoptosis [1, 2]. The nutritional and food  
6 functional importance of sphingolipids have been also disregarded for decades. It has  
7 been reported that dietary supplementation with sphingolipids has diverse physiological  
8 effect, such as improving skin barrier function [3, 4], protecting the colon against cancer  
9 [5, 6] and inhibiting inflammation [7, 8]. Sphingolipids are found in egg, milk, meat,  
10 fish, soybean and so on [9]. Dietary sphingolipids can be hydrolyzed by digestive  
11 enzymes in small intestine, although it is relatively hard to hydrolyze and to absorb  
12 compared with glycerolipids [10-12]. On the other hand, it has been reported that  
13 sphingomyelin (SM), which is a major phosphosphingolipid in animals, inhibits luminal  
14 absorption of cholesterol [13, 14]. One potential mechanism for this suppression may be  
15 associated with SM that may decrease micellar solubilization and transfer of cholesterol  
16 from the micellar matrix to the intestinal cells. In addition, it seems that free sphingoid  
17 bases liberated in intestinal tract may be important for inhibitory effect of dietary  
18 sphingolipids on cholesterol absorption [15]. Plasma cholesterol level is dependent on  
19 several parameters, including endogenous synthesis, secretion, and catabolism of the  
20 various plasma lipoproteins. Other major contributors to the amount of cholesterol  
21 entering the body each day include the amount of cholesterol in the diet and the rate by  
22 which the dietary cholesterol is absorbed [16, 17]. For example, a 90% reduction of  
23 cholesterol absorption in moderately hypercholesterolemic subjects has been shown to  
24 reduce plasma cholesterol and LDL levels by 35% [18].

1           The physiologically active substances including glucosylceramide (GlcCer) and  
2 some related compounds have been extracted from a variety of sea cucumber species  
3 [19, 20]. Dry sea cucumber contains ~200 mg GlcCer per 100 g dry weight [21]. GlcCer  
4 used for food ingredient has been isolated from some plant sources, but their content are  
5 very low (1-40 mg/100 g dry weight) [22]. Thus, sea cucumber might be suitable for  
6 one of dietary source of GlcCer. However, the sphingoid base structures in sea  
7 cucumber are more complicated than those in mammals [23] and there is little  
8 information about food function of these sphingoid bases that are not found in mammals.  
9 The aim of the present study was to evaluate the effect of dietary GlcCer from sea  
10 cucumber on plasma and liver lipids in cholesterol-fed mice.

11

## 12 **Materials and methods**

13

### 14 **Preparation of GlcCer**

15

16 GlcCer were prepared from sea cucumber by a silica gel column after lipid extraction  
17 and saponification as described previously [6, 21]. Their purities were above 96%  
18 determined by HPLC equipped with an evaporative light-scattering detector [22].

19

### 20 **Animals and diets**

21

22 All animals were treated in accordance with the guidelines for the regulation of animals  
23 drafted by the experimentation committee of Kyoto University, Japan. Four-week old  
24 male ICR mice (Japan SLC, Inc, Hamamatsu, Japan) were housed at 25°C with a 12-h

1 light-dark cycle and acclimatized with a commercial diet (MF, Oriental Yeast, Kyoto,  
2 Japan) for one week. Four groups of 8 mice each were submitted to feeding for 2 weeks  
3 with semisynthetic diets (Table 1). Four groups were control diet (C), sea cucumber  
4 GlcCer supplemented diet (S), high cholesterol supplemented diet (HC), and high  
5 cholesterol plus sea cucumber GlcCer supplemented diet (HCS). During the feeding  
6 period, each group of mice was housed with free access to the diet and water. The body  
7 weight and the food intake were measured every day. All prepared diets were stored at  
8 0°C and replaced daily.

9

#### 10 **Sampling procedures**

11

12 At the end of the feeding experiment, mice were sacrificed after blood collection under  
13 light ether anesthesia. Blood was centrifuged at 1,000 g for 15 min at 4°C to separate  
14 plasma. Plasma samples were stored at –80°C until lipid analysis. The liver, spleen  
15 and small intestine were taken, weighed, frozen in liquid nitrogen and kept at –80°C.  
16 A portion of the liver was soaked in RNA later and kept at –80°C for mRNA expression  
17 experiment.

18

#### 19 **Lipid determination**

20

21 Triacylglycerols and total cholesterol of plasma and liver were colorimetrically  
22 determined by commercially available enzyme kits (Wako Pure Chemical, Osaka,  
23 Japan) according to manufacturer's protocol. For liver lipid analysis, the total lipids  
24 were extracted with 2 ml of a mixture of chloroform and methanol (2:1, v/v) from 0.5

1 ml of 25% liver homogenate. The total lipids were dissolved in 1 ml of Triton X-100 before colorimetric assays of the triacylglycerols and cholesterol [24].

3

#### 4 **Determination of mRNA expression of enzymes related to lipid metabolism**

5

6 Total RNA was extracted from the liver of mouse using an RNeasy Mini Kit (QIAGEN,  
7 Valencia, CA, USA) according to the manufacturer's instructions. To quantify mRNA  
8 expression level, real-time quantitative RT-PCR was performed in a BIO-RAD Thermal  
9 Cycler (Bio-Rad, Hercules, CA, USA) using SYBR Green PCR reagents. The following  
10 primers were used: *Fas*, 5'-ACCATGCCAACCTGGTAAAA-3' (sense),  
11 5'-CAGTGTTACAGCCAGGAGA-3' (anti-sense); *Srebp-1c*  
12 5'-GGCTGGCCAATGGACTACTA-3' (sense), 5'-GGCTGAGGTTCCAAAGCAGA-3'  
13 (anti-sense); *Cyp7al*, 5'-AGACCGCACATAAAGCCCGG-3' (sense),  
14 5'-CTTTCATT-GCTTCAGGGCTC-3' (anti-sense); *HmgcoAred*, 5'-  
15 TACAACGCCACGCAGCA-3' (sense), 5'-ACCAACCTTCCTACCTCAGCAA-3'  
16 (anti-sense), and *Ldlr*, 5'-AGCCATTTTCAGTGCCAATC-3' (sense), 5'-  
17 GAGGAGGGCTGTTGTCTCAC-3' (anti-sense). The primer pair of *Gapdh* was  
18 5'-TGGGATCGAGTGAAGGACCT-3' (sense), 5'-CTCCTCCTGCCACTTCTTCTG-3'  
19 (anti-sense). The reaction solution (20 µl final volume) contained 6 µl sample, 10 µl  
20 SYBR Green dye (Bio-Rad Laboratories Inc., Hercules, CA), and 2 µl each primer. The  
21 thermal cycling conditions were as follows: 48°C for 30 min to prevent carrying over of  
22 DNA, an initial denaturation of 95°C for 10 min, followed by 40 cycles of denaturation at  
23 95° C for 15 s and an annealing temperature of 55°C for 1 min.

## 1 **Statistical analyses**

2

3 Data are presented as mean  $\pm$  SD and analyzed by Student's t test or one-way ANOVA  
4 with **Fishier's PLSD** test to identify significant differences between the dietary groups.  
5 A level of  $p < 0.05$  was considered significant.

6

## 7 **Results**

8

9 Dietary sea cucumber GlcCer did not affect the weight of body (**Fig. 1**). **Daily food**  
10 **consumption was similar among the four groups:  $36.5 \pm 4.8$ ,  $33.9 \pm 5.5$ ,  $37.3 \pm 5.1$ ,  $35.5$**   
11  **$\pm 5.2$  g/day/eight mice for C, S, HC and HCS groups, respectively. Based on these data,**  
12 **calculated daily intake of cholesterol in HC and HCS groups were approximately 2.3**  
13 **and 2.2 mg/day/mouse. Liver and spleen weight was increased significantly in case of**  
14 **high cholesterol diet (Table 2). Contrary, the increase of liver and spleen weights was**  
15 **significantly suppressed by dietary GlcCer.**

16 Sea cucumber GlcCer was used to evaluate the effect of GlcCer on plasma and  
17 liver triacylglycerol (TG) and cholesterol concentrations in mice. Dietary sea cucumber  
18 GlcCer without cholesterol supplement increased plasma TG and decreased plasma total  
19 cholesterol (TC) significantly comparing with control group, but liver TG and TC did  
20 not alter significantly (Table 3). Although HCS did not change plasma TG and TC  
21 comparing with HC group, HCS decreased liver TC significantly comparing with HC  
22 group (Table 3).

23 The hepatic expression of five genes was studied by using real-time RT-PCR on  
24 liver samples fed the experimental diet without cholesterol (**Fig. 2**). The mRNA

1 expressions of genes such as *Fas* and *Srebp-1c* involved in fatty acid and TG synthesis  
2 were tended to increase by dietary sea cucumber GlcCer but not significantly. The  
3 mRNA expression of *Ldlr* was significantly increased while *HmgcoAred* showed trend  
4 of increase comparing with control (diet without cholesterol). The gene *Cyp7a1*  
5 involved in bile acids formation was decreased significantly comparing with control.

6

## 7 **Discussion**

8

9 In our results, dietary sea cucumber GlcCer decreased plasma cholesterol concentration  
10 in mice. This cholesterol-lowering effect is possibly, at least in part, mediated through  
11 inhibition of intestinal absorption of both cholesterol and, eventually, would lead to  
12 protection of the liver from cholesterol-induced steatosis. In agreement with this  
13 prediction, dietary GluCer significantly suppressed the increase of liver weight caused by  
14 high-cholesterol diet. Intestinal absorption of cholesterol depends on bile acids and is  
15 favored by the presence of TG-derived fatty acids in the intestine that forms mixture of  
16 micelles with bile acids in which cholesterol is solubilized [13]. It has been reported that  
17 dietary SM inhibits luminal absorption of cholesterol [14]. The formation of stable  
18 cholesterol and SM (or sphingosine) complexes could be the cause of reduced intestinal  
19 absorption of cholesterol. Because of the diversity in chemical structure among the  
20 various sphingolipid species, a wide range of physical and chemical properties are  
21 expected and, thus, the present results might be due to not only specific complex  
22 formation with bile acids or disturbance of bile acids micelles in the intestinal lumen.

23 It was reported that short-term dietary supplements of GlcCer significantly  
24 increased serum SM levels without influence on cholesterol levels in rats [27]. It is



1 known that two types of cholesterol-raising fatty acids in the diet, saturated fatty acids  
2 and trans fatty acids, increase the serum low density lipoprotein cholesterol concentration  
3 [28, 29]. The increase of cholesterol by the sphingolipid-rich diet is more likely caused  
4 by the fatty acids derived from sphingolipids digestion. However, dietary sphingolipids  
5 are relatively hard to hydrolyze and to absorb compared with glycerolipids [10-12].  
6 Indeed, it was also reported that long-term (through two generations) dietary supplements  
7 of sphingolipids could significantly decrease cholesterol (30%) but not SM levels in rats  
8 [30].

9 A reduction in the cholesterol pool in the liver leads to a reduction in bile acid  
10 synthesis as reflected by a reduced expression in the liver of bile acid synthesis gene  
11 *Cyp7a1*, concomitant with an increased expression of genes involved in hepatic  
12 cholesterol synthesis (*HmgcoAred*) and hepatic cholesterol uptake from plasma (*Ldlr*).  
13 To maintain its lipid homeostasis, the liver might compensate for the decrease  
14 sphingolipid-mediated dietary and biliary cholesterol and fatty acids supply from the  
15 intestine by increasing its endogenous cholesterol and fatty acid synthesis, as reflected  
16 in the trend of increased hepatic mRNA concentrations of *HmgcoAred*, *Ldlr*, *Fas* and  
17 *Srebp-1c*. A major regulator of fatty acid synthesis is *Srebp-1c* and it was reported that  
18 cholesterol feeding resulted in a large increase in the expression of *Srebp-1c* mRNA in  
19 the liver of mice [31].

20 In summary, sea cucumber GlcCer supplemented diet significantly decreased  
21 plasma cholesterol in ICR mice. It also decreased liver cholesterol. Further study is  
22 needed to identify the mechanisms of action by sea cucumber sphingoid bases on  
23 intestinal or liver physiology in order to layout the scientific basis for their use in the  
24 prevention of chronic diseases.

1

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4

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23

24

1 **Figure legend**

2

3 **Figure 1.** Body weight of mice during the experimental period.

4

5 **Figure 2.** Effect of sea cucumber sphingolipid on the expression level of *Cyp7a1*,

6 *HmgcoAred*, *Ldlr*, *Fas* and *Srebp-1c* mRNA in mouse liver. Mouse was fed sea

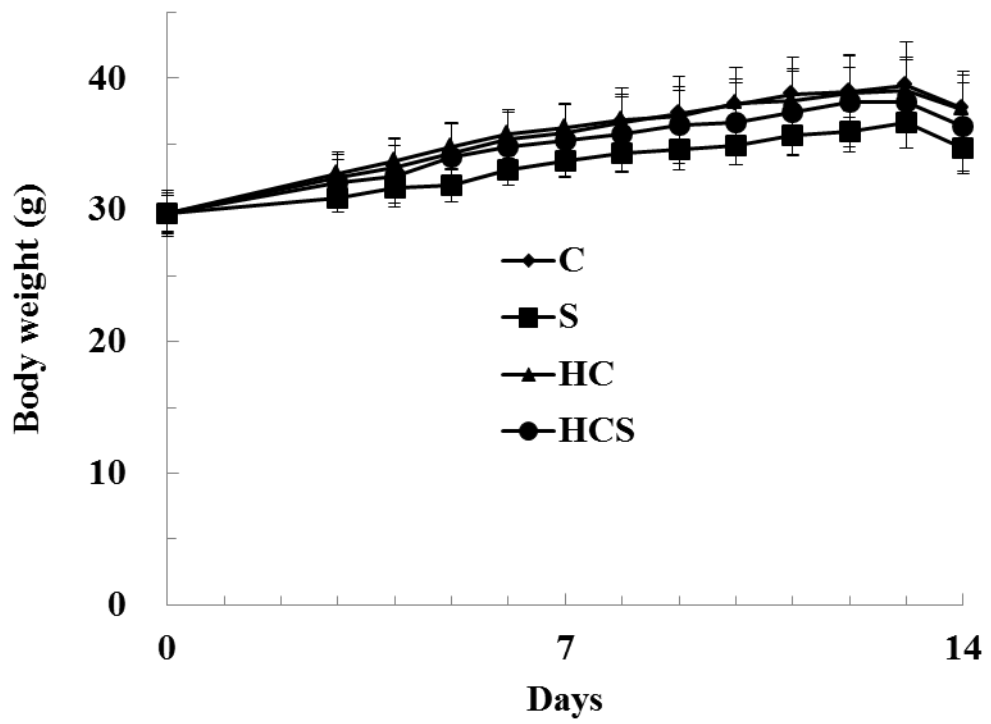
7 cucumber sphingolipid supplemented diet for 2 weeks. Expression of *Cyp7a1*,

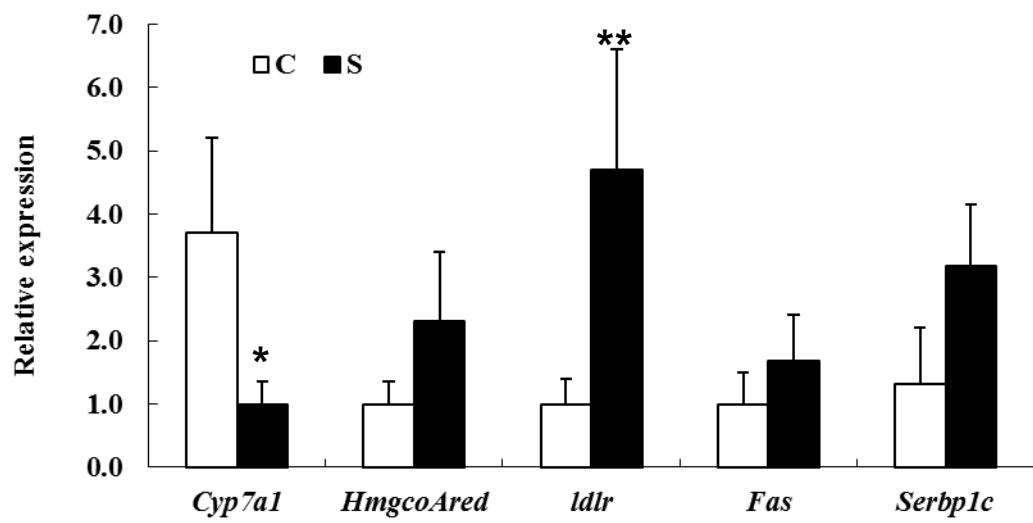
8 *HmgcoAred*, *Ldlr*, *Fas* and *Srebp-1c* was determined by real-time quantitative RT-PCR

9 analysis. Data were normalized to GAPDH mRNA levels and are shown as the means

10  $\pm$ SD. \* $p < 0.01$  and \*\* $p < 0.05$  vs control by Student's-t test

11







**Table 1** Composition of the diets in experiment

Ingredient	C	S	HC	HCS
		g/kg diet		
Cornstrach	397.5	397.5	397.5	397.5
Casein	200.0	200.0	200.0	200.0
Dextrinized cornstrach	132.0	132.0	132.0	132.0
Sucrose	100.0	95.0	92.5	87.5
Soybean oil	70.0	70.0	70.0	70.0
Cellulose	50.0	50.0	50.0	50.0
Mineral mix	35.0	35.0	35.0	35.0
Vitamin mix	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5	2.5
Cholesterol			5.0	5.0
Sodium cholate			2.5	2.5
Sea cucumber SL		5.0		5.0

C, Control diet; S, Sea cucumber sphingolipid supplemented diet; HC, High cholesterol supplemented diet; HCS, High cholesterol + sea cucumber sphingolipid supplemented diet

**Table 2** Effects of dietary sphingolipids on weight of body, liver and spleen for 2 weeks of experimental period

Organs	C	S	HC	HCS
Body	37.69 ± 2.83	34.72 ± 1.96	37.64 ± 2.62	36.31 ± 3.33
Liver	1.44 ± 0.22 <sup>a</sup>	1.40 ± 0.11 <sup>a</sup>	2.35 ± 0.36 <sup>c</sup>	1.91 ± 0.16 <sup>b</sup>
Spleen	0.12 ± 0.01 <sup>a</sup>	0.13 ± 0.02 <sup>a,b</sup>	0.20 ± 0.05 <sup>c</sup>	0.16 ± 0.03 <sup>b</sup>

C, Control diet; S, Sea cucumber sphingolipid supplemented diet; HC, High cholesterol supplemented diet; HCS, High cholesterol + sea cucumber sphingolipid supplemented diet

Values in **rows** with different letters are significantly different by **Fisher's PLSD** test ( $p < 0.05$ ).

**Table 3** Plasma and liver lipids of the animals fed different diets for 2 weeks of experimental period

	Lipids	C	S	HC	HCS
Plasma (mg/dL)	TG	114 ± 40 <sup>b</sup>	161 ± 25 <sup>c</sup>	54 ± 13 <sup>a</sup>	74 ± 17 <sup>a</sup>
	TC	153 ± 31 <sup>b</sup>	114 ± 24 <sup>a</sup>	216 ± 44 <sup>c</sup>	179 ± 43 <sup>b,c</sup>
Liver (mg/g)	TG	39.8 ± 17.0	47.7 ± 27.9	22.0 ± 15.5	38.0 ± 22.3
	TC	3.8 ± 0.6 <sup>a</sup>	2.9 ± 0.7 <sup>a</sup>	36.6 ± 4.7 <sup>c</sup>	32.2 ± 6.9 <sup>b</sup>

C, Control diet; S, Sea cucumber sphingolipid supplemented diet; HC, High cholesterol supplemented diet; HCS, High cholesterol + sea cucumber sphingolipid supplemented diet; TG, Triacylglycerol; TC, Total cholesterol

Values in rows with different letters are significantly different by Fisher's PLSD test ( $p < 0.05$ ).