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Author(s)	Mikami, Nana; Hosokawa, Masashi; Miyashita, Kazuo
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Effects of sea squirt (*Halocynthia roretzi*) lipids on white adipose tissue weight and blood glucose in diabetic/obese KK-*A^y* mice

NANA MIKAMI, MASASHI HOSOKAWA and KAZUO MIYASHITA

Faculty of Fisheries Sciences, Hokkaido University, Hokkaido 041-8611, Japan

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Abstract. Lipids extracted from *Halocynthia roretzi* contain n-3 polyunsaturated fatty acids such as eicosapentaenoic acid and docosahexaenoic acid, as well as carotenoids. The aim of the present study was to evaluate the effect of *H. roretzi* lipids on white adipose tissue (WAT) weight and high blood glucose levels in diabetic/obese KK-*A^y* mice. *H. roretzi* lipids were fed to the diabetic/obese KK-*A^y* mice for 5 weeks. In the mice treated with the *H. roretzi* lipids compared to control mice, WAT weight was reduced, blood glucose levels and leptin mRNA expression in the epididymal WAT were significantly decreased, serum leptin levels also tended to decrease, and serum adiponectin levels tended to increase. These results demonstrate that *H. roretzi* lipids have beneficial health effects on diabetic/obese KK-*A^y* mice.

Introduction

Halocynthia roretzi (Maboya) is an edible marine invertebrate sea squirt with an orange-colored mantle covered by a hard tunic that is eaten in Japan and Korea. *H. roretzi* contains various functional components such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), carotenoids, taurine and plasmalogen (1-3). In a previous study, Kawasaki reported that hexane extracts from *H. roretzi* muscle decreased total cholesterol, triglycerides, phospholipids and NEFA in the serum of rats (4). We also reported that acetylene carotenoids such as alloxanthin and diatoxanthin contained in *H. roretzi* lipids suppress the secretion of proinflammatory cytokines such as interleukin-6 and interleukin-1 β from macrophage-like RAW 264.7 cells stimulated by lipopolysaccharides (5). In addition, halocynthiaxanthin and fucoxanthinol isolated from *H. roretzi* have been reported to induce apoptosis in human leukemia and colon cancer cells (6).

In recent years, the incidence of type 2 diabetes has increased worldwide. Diabetes, a state of high blood glucose level (also known as hyperglycemia), is closely associated with obesity. In hypertrophic adipose tissue in which excessive fat accumulation is observed in obesity, dysregulation of adipocytokine secretion occurs and induces insulin resistance (7). Therefore, it is crucial to prevent type 2 diabetes by ameliorating excessive fat accumulation and adipocytokine production in white adipose tissue (WAT).

In this study, we examined the effects of *H. roretzi* lipids on WAT weight and high blood glucose levels in diabetic/obese KK-*A^y* mice.

Materials and methods

Materials. *H. roretzi* was obtained from Kamiiso Fisheries Cooperative Association (Hokkaido, Japan). Sucrose and L-cystine used in animal diets were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Choline bitartrate and tetra-butylhydroquinone were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Soybean oil was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other ingredients for the animal diets were obtained from Clea Japan, Inc. (Tokyo, Japan).

Extraction of *H. roretzi* lipids. The entire body of *H. roretzi* including the tunic was cut into small pieces, and crude lipids were extracted with ethanol for 4 days at room temperature under shade. The extraction was repeated twice. The pooled extracts were concentrated by evaporation, and water soluble components were removed from crude lipids using chloroform-methanol-water (10/5/3, v/v/v). *H. roretzi* lipids were obtained from the chloroform layer.

Animals. This animal experiment was approved by the Ethics Committee of Experimental Animal Care of Hokkaido University. KK-*A^y* diabetic/obese mice (4-week-old, male) were obtained from Clea Japan, Inc. The mice (n=6) were individually housed at 23 $\pm$ 1°C and 45-60% humidity under a 12-h light/dark cycle. A diet prescribed by the American Institute of Nutrition (AIN-93G) (8) was used as the control diet. The composition of the control diet consisted of 10.0% soybean oil, 37.4% cornstarch, 12.4% dextrised cornstarch, 20.7% casein, 9.4% sucrose, 5.0% cellulose, 3.5% AIN-93G mineral mix, 1.0% AIN-93G vitamin mix, 0.3% L-cystine,

Correspondence to: Dr Masashi Hosokawa, Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan
E-mail: hoso@fish.hokudai.ac.jp

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0.25% choline bitartrate and 0.0014% tetra-butylhydroquinone. After a 1-week acclimatization period with the control diet, the mice were randomly divided into two groups. The experimental group was fed the experimental diet containing 5% soybean oil + 5% *H. roretzi* lipids (5% *H. roretzi* lipid diet). After 5 weeks of feeding, the mice were fasted for 12 h, and blood was collected under anesthesia using diethyl ether. The liver and WAT were immediately excised and weighed. Parts of the epididymal and mesenteric WAT were conserved in RNAlater® (Sigma-Aldrich) to measure the adipocytokine mRNA expression level. The serum was separated from blood and stored at -20°C for measurements of insulin, adiponectin and leptin levels.

Analysis of blood glucose levels. Blood glucose levels were measured using a blood glucose monitor (Glutest Neo Sensor; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan) in mice fed for 33 days with the control diet or the 5% *H. roretzi* lipid diet after 12 h of fasting. This sensor is based on the glucose dehydrogenase method.

Glucose tolerance test. After 27 days of feeding, a glucose tolerance test was performed. Mice were fasted for 4 h, and D-glucose solution was administered (2 mg/g of body weight). Blood glucose levels were measured at 0, 15, 30, 60, 90, 120 and 150 min after glucose administration by a Glutest Neo Sensor.

Measurement of adipocytokine gene expression. Total RNA was extracted from RNAlater-treated epididymal and mesenteric WAT samples (<100 mg) using an RNeasy Lipid Tissue Mini kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. cDNA was synthesized from total RNA by reverse transcription reactions using the High-Capacity cDNA Archive kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Real-time quantitative PCR analysis was performed with an automated sequence detection system (ABI PRISM 7500; Applied Biosystems Japan Ltd.). The cycling conditions of PCR were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. Adiponectin, leptin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression levels were measured by Taq Man Gene Expression assays from Applied Biosystems Japan Ltd. PCR primers (adiponectin, Mm00456425_m1; leptin, Mm00434759_m1; GAPDH, Mm99999915_g1) were also purchased from Applied Biosystems Japan Ltd.

Measurement of insulin, adiponectin and leptin concentrations in serum. Serum insulin, leptin and adiponectin levels were measured using the mouse insulin ELISA kit (H-type), the mouse leptin ELISA kit (Shibayagi Co., Ltd., Gunma, Japan) and the mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), respectively, according to the manufacturer's protocol.

Statistical analysis. Data were expressed as the mean $\pm$ standard error (SE). Statistical significance was determined between the two groups using Welch's t-test. A significant difference was defined at $P < 0.05$ or $P < 0.01$.

Table I. Effect of *H. roretzi* lipids on the growth parameters of KK-*A^y* mice.

	Control	<i>H. roretzi</i> lipids
Final body weight (g)	37.3 $\pm$ 1.2	38.8 $\pm$ 1.0
Change in body weight (g)	10.3 $\pm$ 0.8	11.4 $\pm$ 0.7
Food intake (g/day)	3.9 $\pm$ 0.4	4.4 $\pm$ 0.2

Values are expressed as the mean $\pm$ SE (n=6).

Results and Discussion

The yield of lipids obtained from the entire body of *H. roretzi* was 5.1% yield per dry weight. The lipid composition was 49.1% neutral lipids, 38.4% polar lipids containing carotenoids and 12.5% others. The fatty acid composition of *H. roretzi* lipids was mainly 11.4% palmitic acid, 27.2% EPA and 13.8% DHA, respectively. In addition, alloxanthin, halocynthiaxanthin and diatoxanthin were detected in *H. roretzi* lipids using high performance liquid chromatography as previously reported (2,5).

To investigate the effects of *H. roretzi* lipids on WAT weight and blood glucose level, KK-*A^y* diabetic/obese mice were fed a diet containing 5% *H. roretzi* lipids + 5% soybean oil for 5 weeks. Control mice were fed a diet containing 10% soybean oil. No significant difference in food intake and body weight gain was noticed between the two groups during the experimental period (Table I). However, the WAT weight to body weight was significantly lower in the mice fed the diet containing 5% *H. roretzi* lipids compared to the control mice (Fig. 1). In addition, the 5% *H. roretzi* lipid diet significantly decreased serum LDL-cholesterol levels compared to the control (Fig. 2). Triglyceride, free fatty acids and total cholesterol levels did not differ between the two groups. Kawasaki reported that hexane extracts from *H. roretzi* decreased VLDL and LDL-cholesterol in Wistar rats (4). *H. roretzi* lipids extracted with ethanol had the same effect on the LDL-cholesterol of the diabetic/obese KK-*A^y* mice.

Blood glucose levels were also significantly lower in the mice fed the diet containing 5% *H. roretzi* lipids compared to the control mice after 33 days of feeding and 12 h of fasting (Fig. 3). Furthermore, serum insulin levels in mice fed the diet containing 5% *H. roretzi* lipids tended to decrease compared to the control mice (Fig. 3). To evaluate the insulin sensitivity of the KK-*A^y* mice fed the diet containing 5% *H. roretzi* lipids, we performed a glucose tolerance test. After administration of glucose (2 mg/g of body weight), the blood glucose concentrations of the mice fed the diet containing 5% *H. roretzi* lipids were significantly low at all time intervals as compared to those of the control mice, although the reduction in the blood glucose levels of the 5% *H. roretzi* lipid-fed mice was not significantly different from that of the control mice (Fig. 4). KK-*A^y* mice are known to have hyperglycemia, hyperinsulinemia and hyperleptinemia, and exhibit insulin resistance along with type 2 diabetes. Our results suggest that *H. roretzi* lipids are effective for preventing or improving hyperglycemia through the attenuation of WAT weight gain in KK-*A^y*

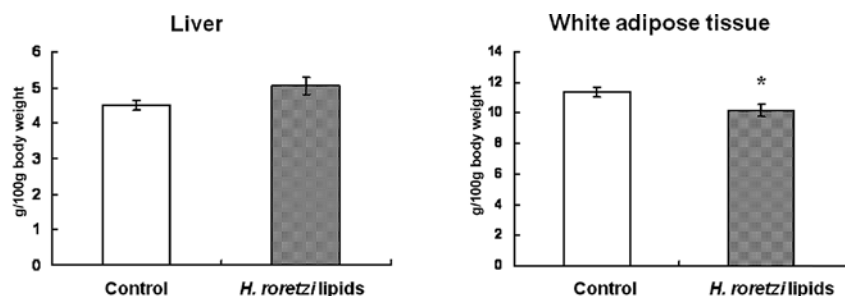


Figure 1. Effect of *H. roretzi* lipids on liver and white adipose tissue weight in KK-*A^y* mice. Mice were fed the control or 5% *H. roretzi* lipid diets for 5 weeks. WAT weight: total weight of epididymal, mesenteric, perirenal, retroperitoneal and inguinal white adipose tissues. Values are expressed as mean $\pm$ SE (n=6). *P<0.05 vs. control.

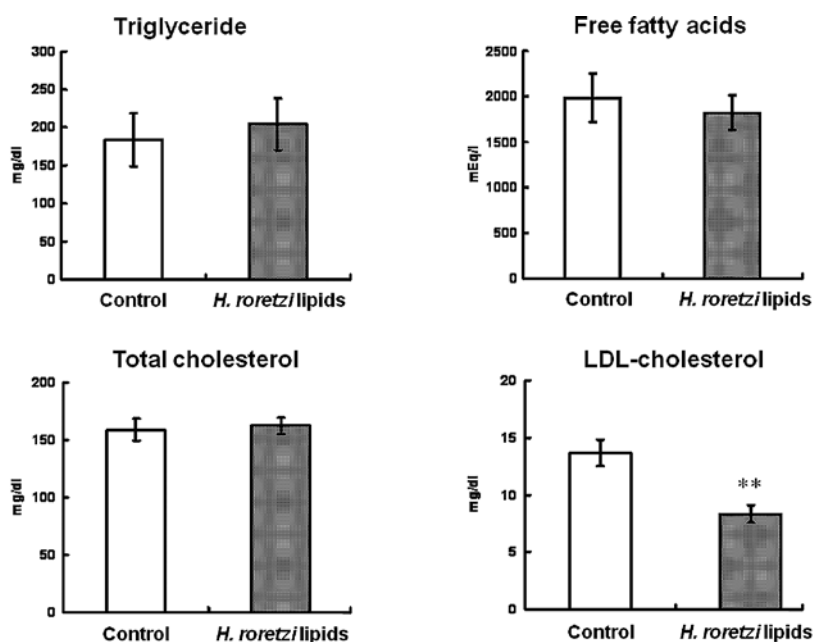


Figure 2. Effect of *H. roretzi* lipids on serum lipid parameters in KK-*A^y* mice. The mice were fed the control or 5% *H. roretzi* lipid diet for 5 weeks. Serum triglyceride, free fatty acids, total cholesterol and LDL-cholesterol levels were measured after 12 h of fasting. Values are expressed as the mean $\pm$ SE (n=6). **P<0.01 vs. control.

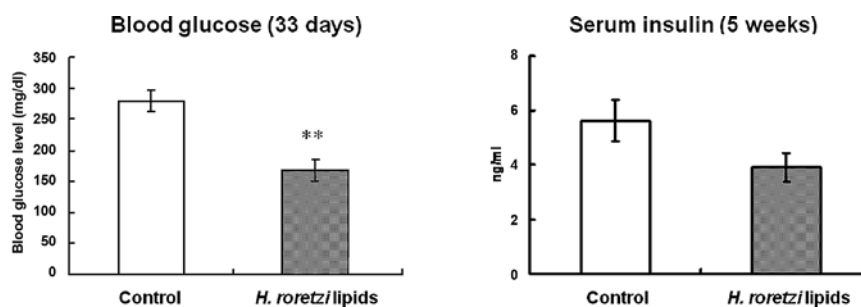


Figure 3. Effect of *H. roretzi* lipids on blood glucose and serum insulin levels in KK-*A^y* mice. Mice were fed the control or 5% *H. roretzi* lipid diet for 5 weeks. Blood glucose levels were measured after 12 h of fasting in mice which had been fed the diets for 33 days. Furthermore, the serum insulin level was measured by ELISA after 12 h of fasting in mice fed the diets for 5 weeks. Values are expressed as the mean $\pm$ SE (n=6). **P<0.01 vs. control.

mice. Adiponectin up-regulates insulin signaling by activating PPAR α and AMP kinase (9). In hypertrophied adipose tissue observed in obese mice, dysregulation of adipocytokine secretion occurs and induces insulin sensitivity. Adiponectin, in particular, is known to be reduced in obese states (10). In

this study, the serum adiponectin and adiponectin mRNA expression levels in mesenteric WAT in the 5% *H. roretzi* lipid diet-fed mice tended to increase, although not significantly, as compared to the control mice (Figs. 5 and 6A). In addition, serum leptin levels tended to decrease and leptin mRNA

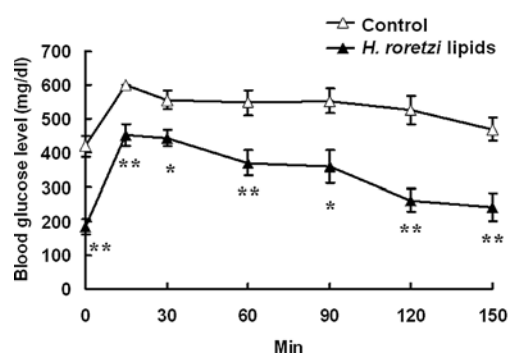


Figure 4. Glucose tolerance test in KK-*A*^y mice fed a diet containing *H. roretzi* lipids. Mice were fasted for 4 h after 4 weeks of feeding and were then orally administered D-glucose solution (2 mg/g of body weight). After 0, 15, 30, 60, 90, 120 and 150 min of administration, the blood glucose levels were measured using the Glutest Neo Sensor. Blood glucose levels >600 mg/dl were calculated as 600 mg/dl, which was the maximum value of the Glutest Neo Sensor. Values are expressed as the mean $\pm$ SE (n=6). *P<0.05 vs. control. **P<0.01 vs. control.

expression levels in epididymal WAT were significantly lower in mice fed the diet containing 5% *H. roretzi* lipids than in the control mice (Figs. 5 and 6B). Serum leptin is known to be correlated positively to fat mass (11). Therefore, the results in the 5% *H. roretzi* lipid group suggest that the serum leptin levels were dependent on the suppression of WAT accumulation in the KK-*A*^y mice.

The fatty acid composition of *H. roretzi* lipids was 27.2% EPA and 13.8% DHA. These n-3 polyunsaturated fatty acids have been reported to suppress fat accumulation in WAT and to decrease high blood glucose levels in genetically induced diabetic/obese mice (12) and high fat-induced obese mice (13). However, the EPA and DHA concentrations in the 5% *H. roretzi* lipid diet were 0.48 and 0.25%, which is lower than in previous reports (12,13). In addition, we did not observe a suppressive effect on WAT weight gain and blood glucose levels in KK-*A*^y mice fed a diet containing 7% fish

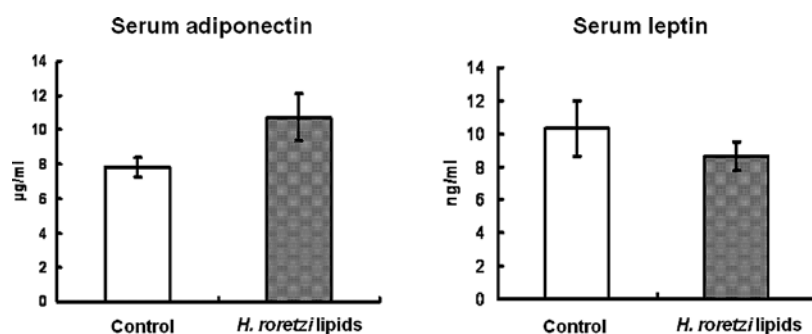
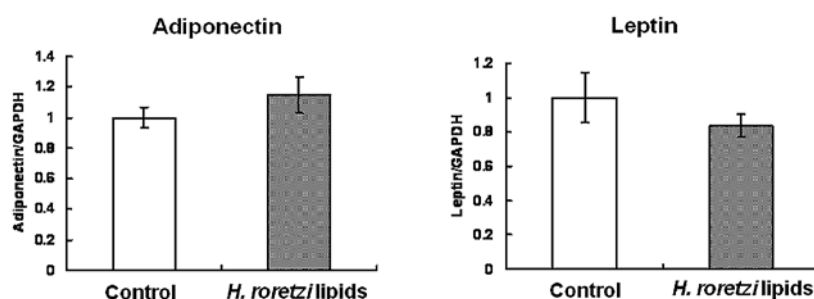


Figure 5. Effects of *H. roretzi* lipids on serum adiponectin and leptin levels in KK-*A*^y mice after 5 weeks of feeding with the control or 5% *H. roretzi* lipid diet. Blood samples were collected after 12 h of fasting, and adipocytokine levels were measured by ELISA. Values are expressed as the mean $\pm$ SE (n=6).

A Mesenteric WAT



B Epididymal WAT

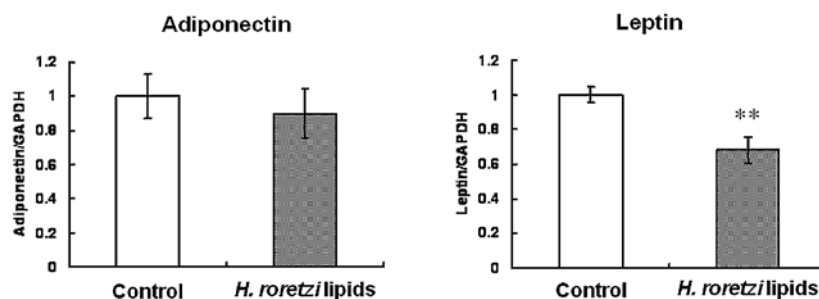


Figure 6. Effects of *H. roretzi* lipids on adiponectin and leptin mRNA expression levels in the mesenteric (A) and epididymal (B) WAT of KK-*A*^y mice after 5 weeks of feeding with the control or 5% *H. roretzi* lipid diet. Values are expressed as the mean $\pm$ SE (n=6). **P<0.01 vs. control.

oil (14). Therefore, the suppressive effects of 5% *H. roretzi* lipids on WAT weight gain and hyperglycemia in the KK-*A^y* mice depended not only on EPA and DHA, but also on other compounds. Furthermore, it is possible that n-3 PUFAs and other compounds had a combined effect. Thus, further investigation is required to clarify the mechanisms of *H. roretzi* lipids.

In conclusion, the present study indicates that *H. roretzi* lipids suppress fat accumulation in WAT and reduce high blood glucose levels in diabetic/obese KK-*A^y* mice. Furthermore, a decrease in serum LDL-cholesterol level was observed in KK-*A^y* mice fed a diet containing 5% *H. roretzi* lipids.

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