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Octopus (*Enterocopus dofleini*) Liver Extract Displays Triglyceride-Lowering Effect in HepG2 Cells

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Abstract: Fatty liver disease is characterized by the accumulation of triglycerides and other fats in the liver cells and is believed to be a risk of later chronic liver disease. Diet is one of the key ways to treat fatty liver disease. Octopus (*Enterocopus dofleini*) liver is eaten in some regions in Japan, but mostly discarded. For utilization of octopus liver, the lipid-lowering effect of octopus (*Enterocopus dofleini*) liver extract was investigated using human hepatoma cells (HepG2 cells). The present study showed that the octopus liver extract reduced the triglyceride content, but not cholesterol content, in HepG2 cells. Treatment with the octopus liver extract increased the mRNA expression of genes for peroxisome proliferator-activated receptor (PPAR)-α, medium chain acyl-CoA dehydrogenase (MCAD), and acyl-CoA oxidase (ACO) associated with β-oxidation. On the contrary, the extract did not change the mRNA expression of genes for sterol regulatory element-binding protein (SREBP)-1, acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)-1, and peroxisome proliferator-activated receptor (PPAR)-γ involved in fatty acid synthesis. These results suggest that octopus liver extract may decrease the triglyceride level in HepG2 cells by promoting β-oxidation, suggestive of its usefulness as a food for lowering triglycerides in liver.

Keywords: Efficient Utilization, Octopus Liver Extract, HepG2 Cells, Triglyceride

1. Introduction

Liver plays a central role in the biogenesis of major metabolites such as glucose, fatty acids, and cholesterol. Excessive alcohol intake causes fatty liver under a condition characterized by large accumulation of triglycerides and other fats in liver cells [1-3]. The accumulation of fatty acid in the liver depends on the balance between the processes of its delivery and removal and may be associated with a decrease in the mitochondrial fatty acid β-oxidation, increase in the endogenous fatty acid synthesis, or enhancement in the delivery of fatty acids to the liver [4]. Fatty liver disease exhibits a gradual progression from simple hepatic steatosis to steatohepatitis. Diet is one of the key ways to treat fatty liver disease. Dietary intake of fishes such as salmon, sardines, and trout, which are rich sources of omega-3 fatty acids, is known to protect the liver against fatty liver and hepatic steatohepatitis [5, 6]. Caffeine intake is associated with a lower risk of fatty liver disease, suggestive of the potential protective role of caffeine [7]. Consumption of green tea rich in high-density catechins is shown to decrease liver fat content and inflammation in fatty liver disease [8].

Octopus arms and body parts are common ingredients in Japanese cuisine. However, the consumption of octopus liver is rare and hence, liver is mostly discarded. The present study investigated the biological activity of octopus liver extract for the efficient utilization and reports its triglyceride-lowering effect in liver using human hepatoma HepG2 cells.

2. Materials and Methods

2.1. Preparation of Octopus Liver Extract

Enterocopus dofleini was collected in Hokkaido and the liver tissues isolated. Liver tissues (200 g) were homogenized in deionized water (500 mL) and centrifuged at 12,000 ×*g* for 15 min. The supernatant was collected and used as the

octopus extract.

2.2. Cell Culture

Human hepatoma HepG2 cells were purchased from RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in modified Eagle's medium (MEM) supplemented with 10% fetal calf serum (FCS) under 95% air and 5% CO_2.

2.3. Cell Viability Assay of HepG2 Cells

HepG2 cells were seeded in a 96-well plate at a density of 4×10^3 cells/well in 100 μL of medium. After 24 h, octopus extract was added to the culture medium at various concentrations. Following treatment with the octopus extract for 96 h, the cell number was quantified using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9]. Briefly, MTT (0.5 mg/mL in phosphate-buffered saline [PBS]) was added to each well and incubated for 4 h at 37°C. The medium was carefully aspirated and 200 μL of 20% sodium dodecyl sulfate (SDS) was added to solubilize the colored product. After 24 h, the absorbance was recorded at 570 nm wavelength using a scanning multi well spectrophotometer.

2.4. Cytotoxicity

Cytotoxicity was assessed by measuring the lactate dehydrogenase (LDH) activity. HepG2 cells were treated with the octopus extract for 96 h. Following treatment, the culture medium (50 μL) was transferred to a 96-well culture plate and the cytotoxicity was measured using an LDH cytotoxicity test kit (Wako).

2.5. Triglyceride and Cholesterol Content in HepG2 Cells

HepG2 cells were seeded in a 96-well plate at a density of 1×10^5 cells/well in 100 μL of medium, followed by treatment with the octopus extract at the indicated concentration. After 96 h, the medium was removed and cells washed twice with PBS. Triglycerides and cholesterol were extracted with hexane: propanol (3:2) solution [10] and the extract dried. Triglyceride and cholesterol contents were measured using the Triglyceride E test and Cholesterol E test commercial kit (Wako, Osaka, Japan).

2.6. Oil red-O Dye Staining

Intracellular lipid accumulation was evaluated with the oil red-*O* dye staining. HepG2 cells were washed with PBS and fixed in 3.7% formaldehyde solution containing PBS at 4°C for 5 min. The fixed cells were washed twice with PBS and stained with 0.16% oil red-*O* solution for 20 min. After removing the oil red-*O* solution, cells were washed thrice with distilled water. The cell-incorporated dye was extracted with 60% isopropanol and the absorbance of the extract measured at 490 nm wavelength.

2.7. Semi-quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted from HepG2 cells incubated in the absence or presence of octopus extract using an RNAiso Plus (Takara, Shiga, Japan), as per the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized from 20 μg of total RNA using oligo (dT) primer and PCR was carried out using 0.05 μg of cDNA template. The specific forward and reverse primers used were as follows: β-actin, 5′-TTGTTACAGGAAGTCCCTTGCC-3′ (forward) and 5′-ATGCTATCACCTCCCCTGTGTG-3′ (reverse); peroxisome proliferator-activated receptor (PPAR)-γ 5′-ACTTATCCTGTGGTCCCCGG-3′ (forward) and 5′-CCGACAGAAAGGCACTTGTGA-3′ (reverse); PPAR-α 5′-TCTTCACGATGCTGTCCTCCT -3′ (forward) and 5′-CTATGTTTAGAAGGCCAGGC -3′ (reverse); medium chain acyl-CoA dehydrogenase (MCAD), 5′-CTACCAAGTATGCCCTGGAAAG-3′ (forward) and 5′-TGTGTTCACGGGCTACAATAAG-3′ (reverse); acyl-CoA oxidase (ACO), 5′-GGGCATGGCTATTCTCATTGC-3′ (forward) and 5′-CGAACAAGGTCAACAGAAGTTAGGTTC-3′ (reverse); sterol regulatory element-binding protein (SREBP)-1, 5′-GGAGGGGTAGGGCCAACGGCCT-3′ (forward) and 5′-CATGTCTTCGAAAGTGCAATCC-3′ (reverse); acetyl-CoA carboxylase (ACC), 5′-GAAGGGCTTATATTGCCTATGACCTTAAC-3′ (forward) and 5′-GGGCAGCATGAACTGGAATT-3′ (reverse); fatty acid synthase (FAS), 5′-ACAGGGACAACCTGTAGTTCT-3′ (forward) and 5′-CTGTGGTCCCACTTGATGAGT-3′ (reverse); and stearoyl-CoA desaturase (SCD)-1, 5′-CCTCTACTTGGAAGACGACATTCGC-3′ (forward) and 5′-GCAGCCGAGCTTTGTAAGAGCGGT-3′ (reverse). The intensity of the amplified bands was estimated using ImageJ software. The mRNA expression levels were normalized to actin mRNA level. The amplification cycles were determined based on the relationship between the amount of PCR product detected and cycle number.

2.8. Statistical Analysis

Each experiment was performed in duplicates or triplicates. Data were combined from at least four repeats and expressed as the mean ± standard deviation (SD). Data were analyzed using Student's t-test.

3. Results and Discussion

3.1. Effects of the Octopus Extract on Viability of HepG2 Cells

The effects of the octopus extract on the viability of HepG2 cells was investigated. Following treatment of HepG2 cells with the octopus extract, the number of viable cells was determined using the MTT assay (Figure 1). The octopus extract did not inhibit cell proliferation and changed cell viability. In addition, investigation of the cytotoxicity of the octopus extract using LDH activity revealed that the extract did not induce any toxicity at a concentration of 0.1 mg/mL. The octopus extract was used at concentrations of 0.02 and 0.1 mg/mL in the following experiments.

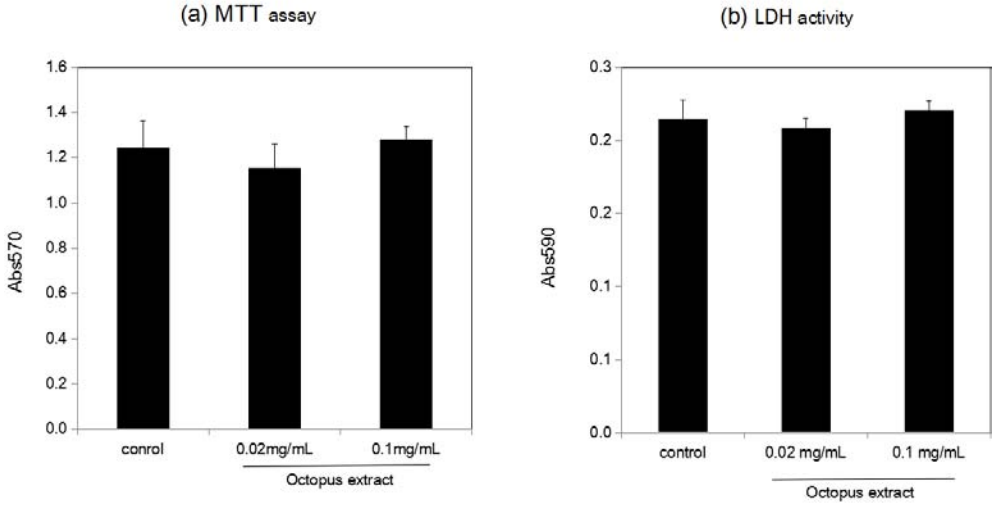

Figure 1. *Toxicity of octopus extract against HepG2 cells. HepG2 cells were treated with the octopus extract for 96 h at the indicated concentration. Cells were cultured in the absence or presence of octopus extract. Data represent results obtained from six wells of a 96-well plate, and the bars represent the means ± standard deviations (SD).*

3.2. Effect of the Octopus Extract on Triglyceride and Cholesterol Accumulation in HepG2 Cells

We investigated the triglyceride and cholesterol content in HepG2 cells in the presence or absence of the octopus extract and found that the extract decreased triglyceride accumulation by about 70% at 0.1 mg/mL concentration as compared with the control (Figure 2a). No change in the cholesterol content was observed in the presence of the extract (Figure 2b). This result was also confirmed by estimating the intracellular lipid accumulation using oil red-*O* dye staining. In presence of the octopus extract, a decrease in the lipid droplet in HepG2 cells was observed as compared with the control. These results suggest that octopus extract may decrease the triglyceride content in HepG2 cells. Next the underlying mechanism of action was investigated by measuring the mRNA expression of genes associated with fatty acid synthesis and β-oxidation.

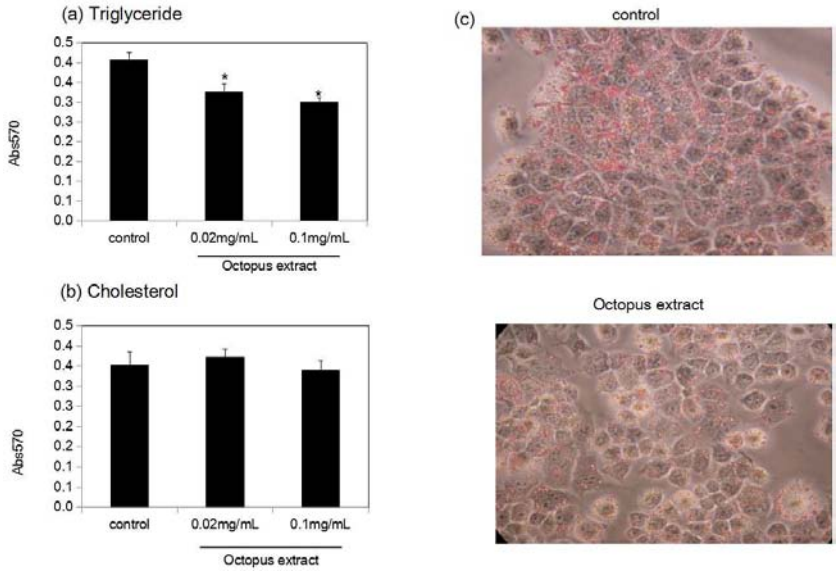

Figure 2. *Effect of octopus extract on triglyceride (a) and cholesterol (b) contents in HepG2 cells. HepG2 cells were treated with the octopus extract at the indicated concentration. After 96 h, triglyceride and cholesterol were extracted and their levels measured. Data represent results obtained from six wells of a 96-well plate, and the bars represent the means ± SD. Statistical significance was determined by Student's t-test: *p < 0.05 relative to control. (c) Lipid droplet in HepG2 cells were stained with the oil red-O dye and photographed under phase-contrast microscopy.*

3.3. mRNA Expression of Genes Associated with Fatty Acid Synthesis and β-oxidation

Lipid accumulation in liver is caused by an increase in the free fatty acid uptake of liver, impaired fatty acid β-oxidation, or increased incidence of *de novo* lipogenesis. We investigated the mRNA expression of genes associated with β-oxidation. Studies suggest that PPAR-α, a member of the

nuclear receptor superfamily of transcription factors, is involved in the regulation of fatty acid β-oxidation [11-13]. Activation of PPAR-α induces an increase in the mRNA expression of its target genes such as ACO and MCAD, thereby resulting in the upregulation of β-oxidation. Treatment of HepG2 cells with the octopus extract resulted in a significant increase in the mRNA expression of PPAR-α, MCAD, and ACO (Figure 3). In particular, a two-fold increase in the expression of ACO was observed following treatment with the octopus extract. Next, we investigated the mRNA expression of genes associated with fatty acid synthesis. SREBP-1 and PPAR-γ affect the transcription of genes such as ACC, FAS, and SCD1, which regulate the *de novo* lipid synthesis [14, 15]. Treatment with the octopus extract did not change the expression of SREBP-1, PPAR-γ, ACC, FAS, and SCD (Figure 4), suggesting that the octopus extract has no effect on the lipid synthesis but promotes β-oxidation activity in HepG2 cells. Lipid content is regulated by a balance between fatty acid synthesis and uptake and β-oxidation. The triglyceride-lowering effect of the octopus extract may be attributed to its ability to upregulate the expression of genes associated with β-oxidation. Triglyceride accumulation has been linked to an increased risk of fatty liver and associated diseases. Octopus liver may be effective as a food for the prevention of fatty liver. Future studies should estimate the *in vivo* triglyceride-lowering effect of octopus extract in the liver.

Polyunsaturated fatty acids, polyphenol, and l-carnitine are known to upregulate the expression of a rate-limiting enzyme in the β-oxidation process in HepG2 cells, leading to a decrease in the plasma lipid concentration [16-18]. Taurine and fish oil decreased the white adipose tissue weight in type 2 diabetic mice through the inhibition of fatty acid synthesis and promotion of β-oxidation in the liver [19]. The β-oxidation stimulatory effect of the octopus extract may be effective in decreasing the fat weight and lowering plasma lipid concentration.

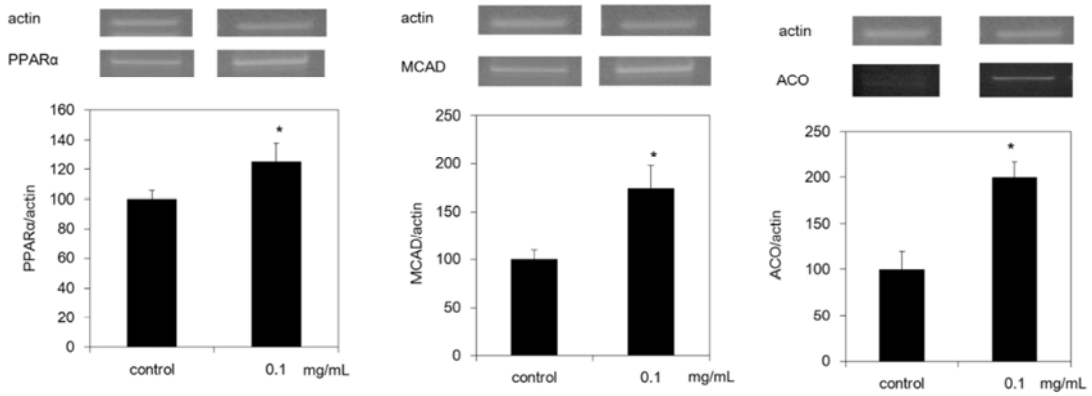

Figure 3. *Semi-quantitative RT-PCR analysis of genes associated with β-oxidation. After treatment of HepG2 cells with the octopus extract at the indicated concentration, total RNA was extracted and semi-quantitative RT-PCR performed. The bars represent the means ± SD. Statistical significance was determined by Student's t-test: *p < 0.05 relative to control.*

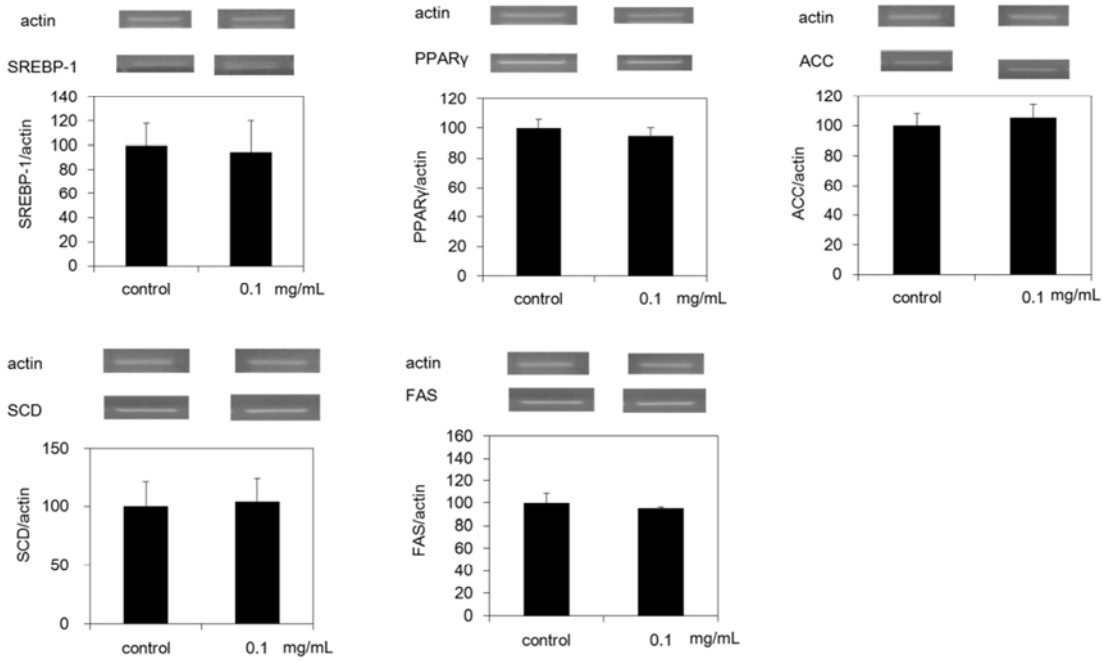

Figure 4. *Semi-quantitative RT-PCR analysis of genes associated with fatty acid synthesis. After treatment of HepG2 cells with the octopus extract at the indicated concentration, total RNA was extracted and semi-quantitative RT-PCR performed. The bars represent the means ± SD.*

4. Conclusion

Results presented here suggest that octopus liver extract may decrease the triglyceride level in HepG2 cells by promoting β-oxidation, suggesting that octopus liver may be effective as a food for the prevention of fatty liver.

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