Cordyceps militaris alleviates non-alcoholic fatty liver disease in ob/ob mice

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BACKGROUND/OBJECTIVES: Non-alcoholic fatty liver disease (NAFLD) is becoming an important public health problem as metabolic syndrome and type 2 diabetes have become epidemic. In this study we investigated the protective effect of Cordyceps militaris (C. militaris) against NAFLD in an obese mouse model.

MATERIALS/METHODS: Four-week-old male ob/ob mice were fed an AIN-93G diet or a diet containing 1% C. militaris water extract for 10 weeks after 1 week of adaptation. Serum glucose, insulin, free fatty acid (FFA), alanine transaminase (ALT), and proinflammatory cytokines were measured. Hepatic levels of lipids, glutathione (GSH), and lipid peroxide were determined.

RESULTS: Consumption of C. militaris significantly decreased serum glucose, as well as homeostasis model assessment for insulin resistance (HOMA-IR), in ob/ob mice. In addition to lowering serum FFA levels, C. militaris also significantly decreased hepatic total lipids and triglyceride contents. Serum ALT activities and tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were reduced by C. militaris. Consumption of C. militaris increased hepatic GSH and reduced lipid peroxide levels.

CONCLUSIONS: These results indicate that C. militaris can exert protective effects against development of NAFLD, partly by reducing inflammatory cytokines and improving hepatic antioxidant status in ob/ob mice.

Keywords: Cordyceps militaris, non-alcoholic fatty liver disease (NAFLD), triglyceride, antioxidant, ob/ob mice

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) refers to a spectrum of liver diseases, which include hepatic steatosis (fatty liver), non-alcoholic steatohepatitis (NASH), and advanced fibrosis and cirrhosis in the absence of chronic alcohol use [1]. NAFLD is the most common cause of liver disease [2]. Although relatively benign, steatosis can progress to NASH, an extreme form of NAFLD, and NASH can eventually develop into liver cirrhosis [3]. NAFLD has also been suggested to potentiate liver damage induced by other factors, including alcohol, toxins, and viruses [4]. NAFLD prevalence is estimated at 15-40% in Western countries and 9-40% in Asia [5].

Although the underlying mechanism of NAFLD is not clear, the ‘2-hit hypothesis’ was proposed to explain the pathogenesis [3,6]. The ‘first hit’ is accumulation of triglycerides in the liver (steatosis), which is strongly associated with insulin resistance. The fatty liver is susceptible to injury mediated by the ‘second hit’, which includes inflammatory adipokines/cytokines, oxidative stress, and mitochondrial dysfunction, leading to steatohepatitis and fibrosis. Insulin resistance is a major pathology underlying the development and progression of NASH [7]. Proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been suggested to play a crucial role in the development of insulin resistance [8].

Because metabolic syndrome and type 2 diabetes are important risk factors for NAFLD [9], NAFLD is becoming an important public health problem as these have become epidemic [10]. Although numerous therapeutic agents have been postulated to treat NAFLD [11], no pharmacological treatment is to date known [12].

The Cordyceps species are entomopathogenic fungi that are used as medicinal mushrooms in eastern Asia [13]. Among them, Cordyceps sinensis (C. sinensis) is the most valued mushroom, which has pharmacological effects that are used in traditional Chinese medicine. However, natural C. sinensis is scarce and highly expensive. Therefore, Cordyceps militaris (C. militaris) is a prominent substitute for C. sinensis due to its similar composition and pharmacological effects to C. sinensis and reasonable price [14].

This research was supported by the Technology Development Program for Agriculture and Forestry (610003-03-1-SB110), Ministry of Agriculture, Food and Rural Affairs, Republic of Korea.

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Received: July 9, 2013, Revised: August 3, 2013, Accepted: August 16, 2013
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C. militaris has shown antioxidant activity in vitro [15,16]. C. militaris extract has free radical scavenging activity and has been reported to have hepatoprotective activity in vitro [17]. C. militaris extract has alleviated oxidative injury in HepG2 cells induced by tert-butyl hydroperoxide (t-BHP) by reducing reactive oxygen species (ROS) generation and thiobarbituric acid reactive substances (TBARS) formation. In addition, C. militaris has demonstrated anti-inflammatory activity in vitro [18]. A hot water extract of C. militaris reduced production of nitric oxide (NO) and secretion of TNF-α and IL-6 induced by lipopolysaccharide (LPS) in macrophages. These findings suggest that C. militaris could play a beneficial role in alleviation of NAFLD by improving oxidative stress and reducing inflammation. However, the beneficial effects of C. militaris on NAFLD have not been fully investigated. Therefore, in this study, the protective effect of C. militaris against NAFLD was investigated in leptin-deficient ob/ob mice, which show obesity, insulin resistance, and hyperglycemia and are used as an animal model of NAFLD [19-22].

MATERIALS AND METHODS

Preparation of water extract of C. militaris

The fruiting bodies of C. militaris were freeze-dried and extracted with 20 times their weight of distilled deionized water for 8 h at 90°C and filtered [23,24]. The extracted solution was evaporated under vacuum at 80°C and the residue was lyophilized using a freeze-dryer (yield 33.1%).

Animals and experimental protocol

All animal experiments were approved by the Animal Resource Center at our university (approval no. 2011-44). Four-week-old male C57BL/6-Lepob/ob mice (n = 16) were obtained from Korea Research Institute of Bioscience and Biotechnology, Ochang, Korea. The mice were housed individually under temperature (24 ± 5°C), humidity (55 ± 5%), and light (12 h light/dark cycle) controlled conditions. After acclimatizing for 1 week, the animals were randomly divided into two groups. The control group was fed a diet containing 1% Alphacel, while the experimental group was fed a diet containing 1% C. militaris water extract in place of Alphacel ad libitum for 10 weeks.

Collection of blood and liver samples

At the end of the experiment, the mice were sacrificed by cardiac puncture following an overnight fast. Blood and liver samples were collected and serum was separated by centrifugation of blood samples at 1,500 g for 15 min. Serum and liver samples were stored at -70°C for further analysis.

Measurement of serum glucose and insulin

Serum glucose levels were measured by an enzymatic method using a commercial kit (Asan Pharmaceutical Co., Seoul, Korea). Insulin levels were determined using radioimmunoassay kits (Linco Co., St. Charles, MO, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was estimated by dividing the product of fasting glucose (mg/dL) and insulin levels (ng/mL) by 405 [26].

Measurement of serum free fatty acid (FFA) and hepatic lipids

Serum FFA was measured using an assay kit as described by the manufacturer (Bioassay System, Hayward, CA, USA). To determine hepatic lipids, a portion of the liver tissue was homogenized in saline using a Teflon homogenizer and total lipid was extracted by the method developed by Folch et al. [27]. Total lipids of the liver were determined by a gravimetric method. The hepatic triglyceride contents were measured by an enzymatic method using a commercial serum triglyceride assay kit (Asan Pharmaceutical Co. Korea).

Measurement of serum alanine transaminase (ALT) and proinflammatory cytokines

Serum alanine transaminase (ALT) activities were measured spectrophotometrically using a commercially available kit (Youngdong Pharmaceutical Co., Yongin, Korea) in accordance with the manufacturer’s instructions. Serum levels of TNF-α, IL-6, and monocyte chemotactic protein-1 (MCP-1) were determined using enzyme-linked immunosorbent assay (ELISA) kits specific for mice (eBioscience, Vienna, Austria).

Measurement of antioxidant parameters in liver

Hepatic TBARS were determined using the method of Ohkawa et al. [28]. A portion of the liver tissue was homogenized in 5 volumes of 10 mM sodium phosphate buffer (pH 7.4). To 0.5 mL of the homogenate, a solution composed of 15% trichloroacetic acid (TCA), 0.4% thiobarbituric acid (TBA), and 2.5% HCl (1 mL) was added. The reaction mixture was incubated at 100°C for 45 min, and then cooled on ice. After centrifugation (1,500g for 15 min), the absorbance of the supernatant was measured at 532 nm. TBARS were expressed as nmol malondialdehyde (MDA)/g liver. The glutathione (GSH) level in the liver was quantified by the method of Ellman [29]. A portion of the liver sample was homogenized in 9 volumes of 0.1 mM phosphate buffer (pH 7.4). After centrifugation (10,000g at 4°C for 30 min), the supernatant (0.5 mL) was mixed with 4.5 mL of 5,5-dithiobis-2-nitrobenzonic acid (DTNB) working solution containing 10 mM DTNB and 0.1 M phosphate buffer (pH 8.0; 1:90, v/v). After incubation at room temperature for 15 min, the absorbance was measured at 534 nm. The protein content was measured using the Bradford method [30]. The level of GSH was expressed as nmol/mg protein.

Statistical analyses

The data are expressed as means ± standard error of the mean (SEM). Student’s t-test was used to identify significant differences between the control and experimental groups, and statistical significance was defined as P < 0.05.

RESULTS

Body weight and food intake

Body weight, food intake, and feed efficiency ratio (FER) of the ob/ob control mice and the mice supplemented with 1% C. militaris water extract are shown in Table 1. The body weight, weight gain, food intake, and FER of the C. militaris group were not significantly different from those of the control group.
Glycemic control
The effects of *C. militaris* on glycemic control and insulin resistance are shown in Table 2. Serum glucose levels were significantly lower in the *C. militaris* group than in the control group (*P* < 0.05). Although insulin levels were not significantly different between the two groups, *C. militaris* supplementation significantly reduced the HOMA-IR value in comparison with the control group (*P* < 0.01).

Hepatic lipids and serum FFA and ALT activities
The hepatic total lipid and triglyceride contents of the *C. militaris* group were reduced by 19.8% and 25.3%, respectively, compared with the control group (*P* < 0.05; Table 3). Serum FFA levels were lower in the *C. militaris* group than in the control group (*P* < 0.05; Table 3). Serum ALT activities were significantly reduced by consumption of *C. militaris* in comparison with the control group (*P* < 0.05).

Serum proinflammatory cytokines
Serum TNF-α levels in the *C. militaris* group were significantly decreased by 17.1% in comparison with the control group (*P* < 0.05; Fig. 1). *C. militaris* supplementation reduced the serum IL-6 by 19.9% in comparison with the control group. Serum MCP-1 levels of the *C. militaris* group tended to be low in comparison with the control group, although the difference was not significant.

Hepatic TBARS and GSH contents
The effects of *C. militaris* on lipid peroxide and GSH concentrations in the liver are shown in Fig. 2. Consumption of *C. militaris* decreased hepatic TBARS by 23.4% and increased GSH levels by 18.7% in comparison with the control group (*P* < 0.05).
DISCUSSION

We determined the effect of *C. militaris* supplementation on development of fatty liver, oxidative stress, and inflammatory cytokine levels to evaluate its benefit for NAFLD in ob/ob mice. These mice have a mutation in the ob gene, which encodes leptin, resulting in hyperphagia and obesity [31]. The hepatocytes of these insulin-resistant mice spontaneously become steatotic, making them a valuable tool for studying NAFLD [32].

Supplementation with 1% *C. militaris* reduced serum glucose and the HOMA-IR, a surrogate parameter of insulin resistance [33] in the ob/ob mouse. This finding is in agreement with previous reports. *C. militaris* extract offered at 1% of the diet improved insulin resistance and hyperglycemia without influencing insulin secretion capacity in 90% pancreatectomized rats [34] and in db/db mice [24].

*C. militaris* extract reduced serum FFA levels in this study. In addition, it decreased hepatic total lipids and triglyceride contents and serum ALT, suggesting alleviation of fatty liver.

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