

# *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- $\alpha$  (TNF- $\alpha$ ) 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.

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## 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- $\alpha$  (TNF- $\alpha$ ) 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].

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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- $\alpha$  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-Lep<sup>ob/ob</sup> 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 acclimating for 1 week, the animals were randomly divided into two groups. The control group was offered an AIN-93G diet [25], while the *C. militaris* group was fed a diet containing 1% *C. militaris* water extract in place of the Alphasel *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- $\alpha$ , IL-6, and monocyte chemoattractant 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-nitrobenzoic 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.

**Table 1.** Body weight, food intake, and feed efficiency ratio in ob/ob mice

	Control	<i>C. militaris</i>
Initial body weight (g)	23.9 ± 1.1 <sup>ns2)</sup>	24.6 ± 1.0
Final body weight (g)	46.7 ± 1.2 <sup>ns</sup>	45.8 ± 1.1
Weight gain (g/day)	0.325 ± 0.019 <sup>ns</sup>	0.303 ± 0.016
Food intake (g/day)	4.56 ± 0.10 <sup>ns</sup>	4.33 ± 0.12
FER <sup>1)</sup> (%)	7.14 ± 0.42 <sup>ns</sup>	7.07 ± 0.50

Five-week-old ob/ob mice were fed an AIN-93G diet or a diet containing 1% *C. militaris* water extract *ad libitum* for 10 weeks. Values represent mean ± SEM (n = 8).

<sup>1)</sup> Feed efficiency ratio (%) = (Body weight gain [g/day]/food intake [g/day]) × 100  
<sup>2)</sup> Not significant

**Table 2.** Serum glucose and insulin levels and HOMA-IR value in ob/ob mice

	Control	<i>C. militaris</i>
Serum glucose (mg/dL)	193.9 ± 11.1	160.3 ± 9.1* <sup>2)</sup>
Serum insulin (ng/mL)	9.98 ± 0.59 <sup>ns3)</sup>	9.09 ± 0.46
HOMA-IR <sup>1)</sup>	4.73 ± 0.28	3.60 ± 0.29**

Five-week-old ob/ob mice were fed an AIN-93G diet or a diet containing 1% *C. militaris* water extract *ad libitum* for 10 weeks. Values represent mean ± SEM (n = 8).

<sup>1)</sup> HOMA-IR = (fasting glucose levels [mg/dL] × fasting insulin levels [ng/mL])/405

<sup>2)</sup> \*  $P < 0.05$ , \*\*  $P < 0.01$

<sup>3)</sup> Not significant

**Table 3.** Hepatic lipids and serum FFA and ALT activities in ob/ob mice

	Control	<i>C. militaris</i>
<i>Liver</i>		
Total lipids (mg/g liver)	257.8 ± 18.7	206.7 ± 14.7*
Triglyceride (mg/g liver)	74.1 ± 6.4	55.4 ± 4.9*
<i>Serum</i>		
FFA (mM)	1.27 ± 0.08	1.05 ± 0.06*
ALT (U/L)	181.4 ± 21.4	126.3 ± 13.8*

Five-week-old ob/ob mice were fed an AIN-93G diet or a diet containing 1% *C. militaris* water extract *ad libitum* for 10 weeks. Values represent mean ± SEM (n = 8). \*  $P < 0.05$

### 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

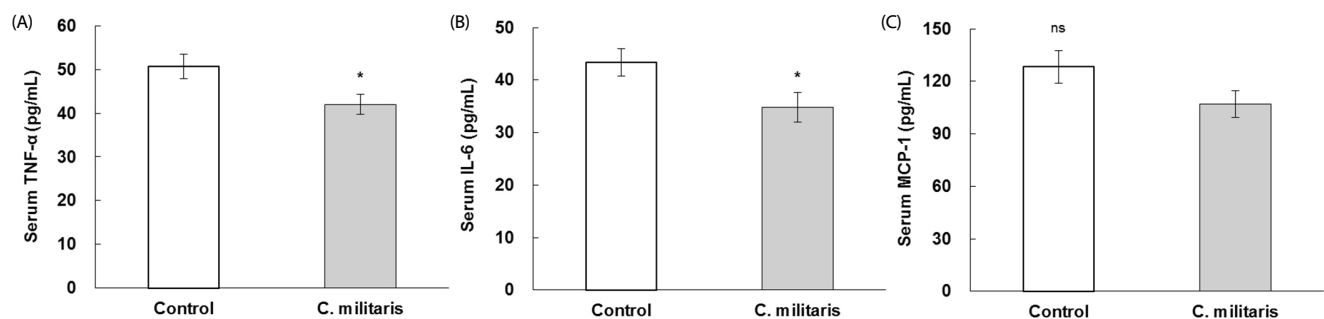
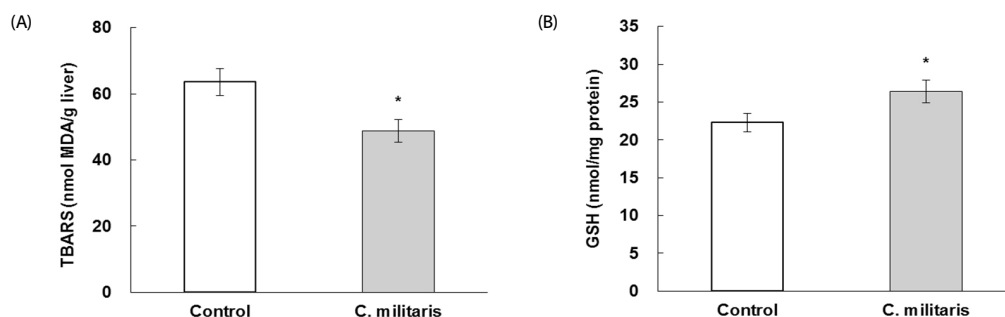
The hepatic total lipid and triglyceride contents of the *C. militaris* group were reduced by 19.8% and 25.3%, respectively, compared with the control group ( $P < 0.05$ ; Table 3). Serum FFA levels were lower in the *C. militaris* group than in the control group ( $P < 0.05$ ; Table 3). Serum ALT activities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

### Serum proinflammatory cytokines

Serum TNF- $\alpha$  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 ( $P < 0.05$ ).

**Fig. 1.** Serum levels of TNF- $\alpha$  (A), IL-6 (B), and MCP-1 (C) in ob/ob mice. Values represent mean ± SEM (n = 8). \*  $P < 0.05$ , <sup>ns</sup>Not significant.**Fig. 2.** Hepatic levels of TBARS (A) and GSH (B) in ob/ob mice. Values represent mean ± SEM (n = 8). \*  $P < 0.05$ .

## 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 mice. 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 and improvement of liver function. Insulin resistance increases secretion of FFAs from peripheral adipose tissue due to enhanced lipolysis [35], resulting in elevated FFA uptake by the liver, which in turn is converted into triglycerides [36]. Reportedly, FFAs in the blood are elevated in animal models of NAFLD [37] and in NAFLD patients [36].

The improvement in insulin resistance by *C. militaris* may be partially related to its effect on proinflammatory cytokines. *C. militaris* decreased serum TNF- $\alpha$  and IL-6 levels. *C. militaris* also showed a tendency to decrease serum MCP-1, although the difference was not statistically significant. These findings are in agreement with a previous report that *C. militaris* reduced TNF- $\alpha$  and IL-6 secretion by LPS-treated murine macrophages [18]. TNF- $\alpha$  mediates insulin resistance by impairing insulin signal transduction [38]. IL-6 affects insulin signaling, leading to development of insulin resistance [39]. MCP-1 contributes to development of the insulin resistance and hepatic steatosis associated with obesity [40]. Thus, the reduction in TNF- $\alpha$  and IL-6 levels induced by *C. militaris* could contribute to improvement of insulin sensitivity and attenuation of hepatic steatosis.

ob/ob mice with fatty liver have been reported to show increased production of ROS, suggesting increased oxidative stress [41]. Steatotic liver is vulnerable to the 'second hit' mediated by oxidative stress, which leads to inflammation [3,6]. In this study, *C. militaris* reduced hepatic TBARS, suggesting alleviation of the oxidative stress in ob/ob mice. *C. militaris* extract was reported to exert a potent antioxidant activity by directly scavenging free radicals [15,16]. In addition, we demonstrated that *C. militaris* elevated hepatic GSH levels in ob/ob mice. *C. militaris* extract has been shown to increase GSH levels in t-BHP-treated HepG2 cells [17]. Ramesh *et al.* reported that cordycepin (3'-deoxyadenosin), one of the major bioactive components of *C. militaris*, elevated hepatic GSH and reduced TBARS in aged rats, suggesting that cordycepin could be the active component in terms of antioxidant activities [42]. Since nuclear erythroid-related factor 2 (Nrf2) plays a key role in synthesis of GSH [43], further study to investigate the effect of *C. militaris* and cordycepin on Nrf2-signaling pathway could be needed to elucidate the mechanism underlying the antioxidant

activity. GSH is an antioxidant involved in scavenging ROS and removing lipid peroxides [44]. Thus, *C. militaris* could contribute to amelioration of the progression of NAFLD by decreasing oxidative stress.

In conclusion, *C. militaris* effectively reduced serum glucose and FFA levels and improved insulin sensitivity in ob/ob mice. *C. militaris* also reduced hepatic triglyceride accumulation and serum ALT. In addition, *C. militaris* ameliorated hepatic oxidative stress and reduced serum proinflammatory cytokine levels. Therefore, *C. militaris* may be beneficial in preventing NAFLD.

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