Effect of *Cordyceps militaris* extract and active constituents on metabolic parameters of obesity induced by high-fat diet in C58BL/6J mice

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\textbf{Abstract}

Ethnopharmacological relevance: *Cordyceps* species which is well-known as 'winter worm summer grass' has long been used as tonics and stimulants to enhance energy, exhibiting a potential for energy metabolism. Clinical trials have suggested their beneficial effect on lipid metabolic disorders such as hyperlipidemia.

Materials and methods: The effect of *Cordyceps militaris* on metabolic parameters was investigated using C58BL/6J mice induced by high-fat diet (HFD). The effect was first determined by assessing the body and organ weight. For further investigation, sections of epididymal adipose tissue were stained with hematoxylin and eosin and the size of epididymal adipocyte was measured by Image analysis system. Fat accumulation in frozen liver sections was assessed by the Oil Red O staining and the plasma biochemical parameters were also assessed. Active constituents were characterized using chromatographic and spectroscopic analysis.

Results: The administration of *Cordyceps militaris* extract (CE) at the dose of 100 mg/kg and 300 mg/kg reduced body weight gain and food efficiency ratio induced by HFD. The amount of epididymal fat and size of adipocytes were also decreased by CE treatment. In addition, liver weight and fat deposition in liver were dramatically reduced in CE-treated group. The treatment of CE also showed beneficial effects on plasma parameters related to lipid profiles. Further study for the characterization of active constituents of Cordyceps resulted in the isolation of two new compounds such as cordyroles A (1) and B (7) together with 12 known compounds including pyrrole alkaloids and nucleotide derivatives. Among the isolated compounds, cordyrole A significantly inhibited adipocyte differentiation and pancreatic lipase activity, whereas cordyrole B was more effective at inhibiting pancreatic lipase. Cordycepin, a characteristic compound of *Cordyceps militaris*, decreased the rate of adipocyte differentiation.

Conclusion: Treatment of CE inhibited HFD-induced metabolic disorders, mainly by improvement in metabolic parameters. As active constituents, pyrrole alkaloids and nucleotide derivatives were characterized. These results suggested that *Cordyceps militaris* might be beneficial for the treatment of metabolic disorders obesity through the combined actions of diverse constituents.

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1. Introduction

Obesity stems from a prolonged imbalance between the levels of energy intake and expenditure, with the surplus being stored as body lipids. Metabolic disorders such as diabetes, atherosclerosis and liver diseases are closely related to obesity (Kopelman, 2000). Fatty liver is the abnormal accumulation of triglycerides in the cytoplasm of hepatocytes and further progresses to steatohepatitis and advanced stages of liver diseases (Dai et al., 2013). Genetic and environmental factors play a role in the development of obesity and the diet is known to be one of the main environmental factors. Especially, increased fat intake, in part due to Western diet, is highly associated with body weight gain which can lead to obesity and other related metabolic diseases. For the study of obesity and the development of anti-obesity therapeutics, diverse animal
model of obesity has been developed using physiologically, genetically and pharmacologically modification (Speakman et al., 2008). Among them, high-fat diet (HFD) feeding in rodent can induce obesity and metabolic disorders that resemble the human metabolic syndrome (Buettner et al., 2007). Therefore, HFD induced animal model is widely used for the development of anti-obesity therapeutics.

Cordyceps species has its trivial name as ‘winter worm summer grass’ in Asian country, because of its different appearance in winter and summer. Its characteristic feature, a pathogenic complex of the fungus (Cordyceps militaris which belongs to Hypocreaceae Family) and the caterpillar (Hepialis armoricans which belongs to Hepiali- dae), enable to produce diverse skeleton of biologically active compounds. Cordyceps has long been used as tonics and stimulants to enhance energy, which exhibited a potential for energy metabolism (Panda and Swain, 2011). Many recent studies and clinical trials have suggested their beneficial effect on lipid metabolic disorders and its related disorders such as hyperlipidemia and hyperglycemia (Paterson, 2008). Many commercial products are available in the market as neutraceuticals.

Cordyceps is well known as a rich source of biologically active components. Nucleosides and polysaccharides are reported as major characteristic constituents of Cordyceps species. Cordyceps have been used for treatment of several diseases such as cancer, fatigue, hyposexualities (Koh et al., 2003; Lee et al., 2006; Kim et al., 2010). Related to metabolic disorders, favorable roles of Cordyceps militaris in the regulation of obesity and diabetes have been reported (Yun et al., 2006; Shimada et al., 2008; Liu et al., 2011). In the present study, the effect of Cordyceps militaris extract (CE) was evaluated using high-fat diet (HFD)-induced obese mice. Characterization of active constituents was also accomplished by chromatographic and spectroscopic analysis.

2. Materials and methods

2.1. Materials

Dried Cordyceps militaris samples were identified and provided by Rural Development Administration in November 2009. Cordyceps militaris samples were produced as previously reported (Choi et al., 1999; Sung et al., 2002; Hong et al., 2010). Briefly, hyphal body suspension of Cordyceps militaris was cultured in rice bran medium. After incubation, fruity bodies from rice bran medium were harvested. Voucher specimen was deposited in the herbarium of College of Pharmacy at Chungbuk National University (CBNU200911-CM). For the preparation of extract, Cordyceps militaris was pulverized and extracted with 50% ethanol for 2 days. The filtrate was evaporated in vacuo, which resulted in Cordyceps militaris extract (CE) for further investigation.

2.2. In vivo anti-obesity effect of Cordyceps extract

2.2.1. Animal treatment

Male C57BL/6j mice (n=60, 4 weeks old, 15 ± 10% g) were purchased from Central Lab. Animal Inc., Seoul, Korea. All mice were housed in a room with controlled temperature (21–23 °C), humidity (55–60%), and lighting (12 h light/dark cycle), and given water ad libitum. After acclimation for 1 week, mice were randomly divided into four groups (n=15/group), normal diet (ND), high-fat diet control (HFD), HFD+CE100 (100 mg/kg of CE), HFD+CE300 (300 mg/kg of CE). Normal diet (ND) group was fed with normal diet, and HFD, HFD+CE100 and HFD+CE300 groups were fed with high fat diet (Table 1) ad libitum. CE sample was diluted in distilled water (DW) and administered orally to mice to HFD+CE100 and HFD+CE300 groups, and DW was administered orally to ND and HFD groups. At the end of experiment, mice were anesthetized and blood, organs were collected. The protocol for this study was approved by the Animal Care and Use Committee of Chungbuk National University (Approved no. CBNUA-370–11-01).

2.2.2. Histopathology

Histological photograph of adipose tissue was analyzed based on the paraffin method using a light microscope. Epididymal adipose tissue was fixed with 10% neutral buffered formalin and embedded in paraffin block. Six μm sections were cut and mounted on glass slide. Paraffin was removed with xylene and alcohol. The sections were then stained with hematoxylin and eosin (H&E). After dehydration by alcohol, the photograph was taken with light microscope. The size of epididymal adipocyte was calculated by Image analysis system (IPKR-1003, Saramsoft Co., Ltd., Korea). For the detection of lipid deposition in liver, liver section were prepared from frozen liver and stained with Oil Red O as previously reported (Fowler and Greenspan, 1985).

2.2.3. Serum biochemical parameters

All mice were fasted for 12 h before sacrifice. Blood was collected and serum was obtained by the centrifugation at 3000 rpm for 10 min at 4 °C. The content of triglyceride (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), alanine transaminase (ALT), aspartate transaminase (AST) and alkaline phosphatase (ALP) were determined using Hitachi7080 analyzer.

2.3. Characterization of active constituents

2.3.1. Extraction and isolation of compounds.

Cordyceps (1.5 kg) was extracted twice with 50% EtOH, which yielded the ethanol extract (93.5 g). The ethanol extract was suspended in H2O and partitioned successively with CH2Cl2, EtOAc, and n-BuOH. The CH2Cl2 fraction (18.9 g) was subjected to column chromatography (MPLC) over silica gel and eluted with hexane-EtOAc–MeOH to give 22 subfractions (CORM1–CORM22). CORM16 was subjected to column chromatography over Sephadex LH-20 and eluted with CH2Cl2–MeOH to yield four subfractions (CORM16A–CORM16D). Compounds 3 (1.9 mg) and 4 (4.0 mg) were obtained from CORE6 and CORM8.

### Table 1

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>ND</th>
<th>HFD</th>
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<tbody>
<tr>
<td>Protein</td>
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<td>24</td>
</tr>
<tr>
<td>Carbohydrate</td>
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<td>41</td>
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<tr>
<td>Fat</td>
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<td>24</td>
</tr>
<tr>
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<tr>
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<td>11.7</td>
</tr>
<tr>
<td>Sucrose</td>
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<td>20.1</td>
</tr>
<tr>
<td>Cellulose</td>
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<td>5.8</td>
</tr>
<tr>
<td>Soybean oil</td>
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</tr>
<tr>
<td>Lard</td>
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</tr>
<tr>
<td>Cholesterol</td>
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<td>0.5</td>
</tr>
<tr>
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<td>1.2</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
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</tr>
<tr>
<td>Calcium carbonate</td>
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<td>0.6</td>
</tr>
<tr>
<td>Potassium citrate</td>
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<td>1.9</td>
</tr>
<tr>
<td>Vitamin mixture</td>
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<td>1.2</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.3</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Unit: %
respectively, by semi-preparative high pressure liquid chromatography (HPLC) and eluted with MeCN-water and MeOH-water. The n-BuOH fraction (67.9 g) was subjected to HP-20 column chromatography with mixture of MeOH-water (0, 20, 40, 60, 80, and 100% MeOH in water) to give six fractions (CORB1–CORB6). The CORB2 fraction was subjected to silica gel MPLC and eluted with CH2Cl2 to yield ten subfractions (CORB2A-CORB2J). Compound 5 (3.0 mg) was obtained from CORB2C by semi-preparative HPLC and eluted with MeOH. Compound CORB3A (1.6 mg) was obtained from CORB3A by column chromatography over Sephadex LH-20 eluted with MeOH, followed by semi-preparative HPLC eluted with MeOH-water. Compound CORB3B (2.1 mg) was obtained from CORB3A by column chromatography over Sephadex LH-20 eluted with MeOH, followed by semi-preparative HPLC eluted with MeOH-water. Compound CORB3C (1.4 mg) was obtained from CORB3C by column chromatography over Sephadex LH-20 eluted with MeOH, followed by semi-preparative HPLC eluted with MeOH-water.

2.3.2. Spectroscopic data of the new compounds
Cordyrrrole B (7): White amorphous powder; UV (MeOH) λ<sub>max</sub> 265 nm; IR<sub>max</sub> 3316, 2927, 1730, 1617 cm<sup>−1</sup>; 1H NMR (CD3OD, 100 MHz) δ 8.28 (1H, s, H-7), 8.26 (1H, s, H-2), 5.98 (1H, d, J = 7.6 Hz, H-1″), 4.76 (1H, dd, J = 5.2, 6.4 Hz, H-2″), 4.35 (1H, m, H-3″), 4.32 (2H, t, J = 5.6 Hz, H-2′), 4.19 (1H, dd, J = 2.5, 5.2 Hz, H-4″ a), 3.91 (2H, m, H-1′), 3.90 (1H, dd, J = 2.4, 12.8 Hz, H-5″). 3.76 (1H, dd, J = 2.4, 12.8 Hz, H-4′), 2.05 (3H, s, COCH₃) ppm; 13C NMR (CD3OD, 100 MHz) δ 152.0 (C-2), 148.2 (C-4), 115.6 (C-5), 154.9 (C-6), 140.2 (C-7), 39.1 (C-1′), 62.8 (C-2′), 171.4 (COCH₃), 19.3 (COCH₂), 89.8 (C-1″), 74.0 (C-2″), 71.2 (C-3″), 86.8 (C-4″), 62.1 (C-5″) ppm; ESIMS (positive mode) m/z: 354 [M + H]+; HRESI-TOF-MS (positive mode) m/z: 354.1404 (calc for C₁₄H₂₀N₂O₆ 354.1414).

2.4. In vitro activity of compounds

2.4.1. Pancreatic lipase activity
Pancreatic lipase inhibitory activity was evaluated using a method reported previously (Kim et al., 2012). The enzyme solution was prepared by reconstituting porcine pancreatic lipase, and pancreatic lipase activity was determined by measuring the hydrolysis of p-nitrophenyl palmitate at 405 nm using a microplate reader. Relative pancreatic lipase activity (%) was calculated as (activity of compound with substrate – negative control of without compound)/(activity of without compound and with substrate – negative control of without compound and substrate) × 100.

Fig. 1. Effect of CE100 and CE300 on body weight (A), FER (C) and organ weight (C) in HFD-induced obese mice. FER was calculated as total weight gain/total food intake. Results are expressed as the mean ± SE (n = 12–15). * p < 0.05 compared with ND group. # p < 0.05 compared with HFD group.
2.4.2. Adipocyte differentiation

3T3-L1 mouse embryo fibroblasts (ATCC CL-173) were obtained from the American Type Culture Collection (Manassas, VA). The cells were stimulated to differentiate with differentiation medium containing DMEM with 10% FBS, 0.5 mM 3-isobutyl-1-methyl-xanthine, 1 μM insulin and 1 μM dexamethasone for 2 days (day 2). Cells were then maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1 μM insulin for another 2 days (day 4), followed by culturing with DMEM with 10% FBS for an additional 4 days (day 8). The cultures were treated with 100 μM isolated compounds for the entire culture period (days 0–8). Lipid droplets in cells were stained with Oil Red O. The Oil Red O stain was dissolved in isopropyl alcohol and optical density was measured at 520 nm using ELISA plate reader for the quantitative analysis (Choi et al., 2011).

2.5. Statistical analysis

The evaluation of statistical significance was determined by Levene’s test followed by one-way ANOVA or Turkey HSD-test with a value of \( p < 0.05 \) considered to be statistically significant.

3. Results and discussion

3.1. In vivo effect of the CE on HFD-induced obesity

The effect of CE on obesity was investigated using HFD-induced male C58BL/6J mice. Diet-induced obesity in rodents has been used as an animal model to investigate environmental effect. HFD-fed rodents become obese and show distinctive symptoms such as increase of adipose tissue, disturbance of lipid metabolism, hyper-insulinemia and fatty liver, which are typically associated with human obesity (Sclafani and Springer, 1976). In our study, the body weight of HFD group was significantly increased compared to that of ND after 2 weeks diet. However, the body weight gains of HFD + CE100 and HFD + CE300 groups were significantly reduced compared to HFD groups, after 21 days and 10 days treatment, respectively (Fig. 1A). On day 42, at the end of experiment, the body weight gains of CE100 [4.29 g] and CE300-treated groups [4.01 g] were reduced dramatically compared to HFD group [8.39 g]. The relative ratio of epididymal fat was also significantly increased in HFD group compared to ND group (Fig. 2A). Treatment of CE100 and CE300 significantly reduced the relative ratio of epididymal fat in dose related manner. The liver weight in HFD group was significantly increased compared to ND group in our study. However, the increase of liver weight induced by high fat diet was significantly attenuated in CE100 and CE300 groups compared to HFD group. The treatment of CE100 and CE300, however, did not affect the relative weight spleen and heart whereas relative weight of kidney was slightly reduced (Fig. 1C).

Food intake was higher in the ND group than in HFD group, but did not differ significantly among the HFD, HFD + CE100 and HFD + CE300 groups. However, food efficiency ratio (FER) of HFD + CE100 and HFD + CE300 groups were significantly reduced compared to HFD group (Fig. 1B).

High ratio of fat consumption accompanies excessive growth of adipose tissue in both cell number and cell size, and consequently induces fat accumulation. As shown in Fig. 2A, the size of adipocyte in epididymal tissue was greatly increased in HFD group, as analyzed by image analyzer after H&E staining. However, the size of adipocyte in CE100 and CE300-treated groups was dramatically decreased compared to HFD (Fig. 2B). HFD diet also induced fat accumulation in liver, which was easily observed by Oil Red O staining (Fig. 2C). However, treatment of CE100 and CE300 greatly reduced fat accumulation in liver. Especially, fat accumulation in HFD + CE300 group was almost completely

Fig. 2. Effect of CE100 and CE300 on lipid parameters in HFD-induced obese mice. (A) Sections of epididymal adipose tissue stained with H&E (magnification, 200×). (B) Quantitation of the size of epididymal adipocyte by Image analysis system. (C) Fat accumulation in frozen liver sections stained with Oil Red O. (D) Plasma biochemical parameters. Data are expressed as mean ± SD (n = 12–15 per group). ALP, alkaline phosphatase (IU/l); AST, aspartate transaminase (IU/l); ALT, alanine transaminase (IU/l); HDL, high-density lipoprotein (mg/dl); LDL, low-density lipoprotein (mg/dl); TC, total cholesterol (mg/dl); TG, triglyceride (mg/dl); \(^*p<0.05\) compared with ND group, \(^#p<0.05\) compared with HFD group.
reduced comparable to ND group. These result shows that CE100 and CE300 efficiently inhibit fat accumulation in liver and epididymal adipocyte tissues.

Persistent high fat and cholesterol diets cause damage to liver, which results in change of enzymes and lipid profiles. HFD caused elevation of plasma level of ALP, AST and ALT, which was recovered in HFD + CE100 and HFD + CE300 groups. In addition, HFD-induced increases of TC, LDL and TG were significantly decreased in CE100 and CE300-treated groups. HDL, however, was decreased in HFD group, which was significantly recovered in CE100 and CE300-treated group (Fig. 2D).

3.2. Structural elucidation

3.2.1. Structural determination of new compounds

Compound 1 was obtained as a pale brown amorphous powder with a molecular formula of C13H10N2O6, determined by HRESIMS (m/z 245.0910 [M + Na]+; calc 222.1004). The IR spectrum showed the presence of an amine group (3345 cm⁻¹) and carbonyl group (1645 cm⁻¹). The maximum UV absorption was observed at 293 nm, which is characteristic of a pyrrole alkaloid (Chin et al., 2003). The presence of pyrrole ring was also supported by the δH 3.65 (1H, m), 3.35 (1H, m) and δC 28.8 (1H), 22.0 (1H), 170.5 (C-6), 143.9 (C-2) in the 1H and 13C NMR spectra. The positions of the aldehyde and hydroxymethyl moiety were C-2 and C-5, respectively, by the HMBC correlation between δC 9.34 (H-1) and δC 143.9 (C-2), and between δH 4.66 (H-6) and δC 132.0 (C-5). Based on these data, the structure of 1 was determined as shown in Fig. 3 and named cordyrrole A.

Compound 7 was obtained as a white amorphous powder with a molecular formula of C14H19N5O6, determined by HRESIMS (m/z 354.1408 [M + H]+; calc 354.1414). The 1H- and 13C NMR spectra of 7 showed characteristic signals for adenosine, a major constituent of Cordyceps (Furuya et al., 1983). The 1H and 13C NMR of 7 also showed two methylene signals at [δH 3.91 (2H, m) and 4.32 (2H, t, J = 5.6 Hz)] and [δC 39.1 and 62.8], which was very similar to those of N6-(2-hydroxyethyl)adenosine. However, additional signals for an acetyl moiety were observed at [δC 20.5 (3H, s), δC 19.3 and 171.4] in the HMBC spectrum, which suggested 7 as an acetylated form of N6-(2-hydroxyethyl)adenosine. The position of an acetyl moiety was determined from the HMBC correlation between δC 171.4 (COCH3) and δH 4.32 (H-2). Taken together, the structure of 7 was determined as shown and was named cordyrrole B.

3.2.2. Identification of known compounds

Twelve known compounds were identified, by the spectroscopic data analysis and comparison with literature values including 5-(hydroxymethyl)-1-(2-oxopiperidin-3-yl)-1H-pyrole-2-carbaldehyde (2), dihydouracil (3), uracil (4), nicotinamide (5), N6-(2-hydroxyethyl) adenosine (6), cordycepin (8), adenosine (9), 2′-O-methyladenosine (10), xanthosine (11), 2′-deoxyuridine (12), uridine (13), thymine (14)


