Natural Product Sciences
9(4) : 291-298 (2003)

Anti-diabetic Effects of CCCA, CMESS, and Cordycepin from *Cordyceps militaris* and the Immune Responses in Streptozotocin-induced Diabetic Mice

Yunha Yun[1], Shinha Han[1], Seungjeong Lee[2], Sung Kwon Ko[3], Chong-kil Lee[4], Nam-Joo Ha[1], and Kyungjae Kim[1]*

[1]*Department of Pharmacy, Sahmyook University, Seoul 139-742, Korea*
[2]*CM Biotec, Kangnung 210-933, Korea*
[3]*Korea Ginseng Institute, Chung-Ang University, Seoul 456-756, Korea*
[4]*College of Pharmacy, Chungbuk National University, Cheongju 360-763 Korea*

Abstract – Anti-diabetic effect of various fractions of *Cordyceps militaris* (CM), CCCA (crude cordycepin containing adenosine), CMESS (ethanol soluble supernatant), and cordycepin were evaluated in streptozotocin (STZ) induced diabetic mice. CMESS showed potent inhibitory activity of 34.7% in starch-loaded mice (2 g/kg) while acarbose as a positive standard exhibited 37.8% of inhibition rate. After 3 days administration (50 mg/kg), CMESS reduced blood glucose level by 35.5% (acarbose, 37.2%). However CCCA, cordycepin, and tryptophan showed no significance. After 7 days administrations for the long-term usage of these drugs, CMESS (50 mg/kg), cordycepin (0.2 mg/kg), and acarbose (10 mg/kg) dramatically reduced blood glucose level (inhibition ratio: 46.9%, 48.4% and 37.5% respectively). CCCA that has high contents of cordycepin (0.656 mg/4 mg) did not influence on reducing blood glucose level. The proliferation of splenocytes and peritoneal macrophages derived from STZ-induced diabetic mice administered samples were evaluated out by addition of mitogens to see the stability of the usage of these herbal medicines. Proliferation of T-lymphocyte was significantly decreased; while NO production was increased more than two fold to STZ control in the cordycepin-administered group. Proliferation of macrophages and NO production were significantly decreased in CMESS administered group. Changes of serum enzyme levels of glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) were also evaluated. Cordycepin administered group was appeared to have more higher levels than those of control in both two enzymes, but it was not significant as compared to acarbose. We conclude that CMESS and cordycepin may be useful tools in the control of blood glucose level in diabetes and promising new drug as an anti-hyperglycemic agent without defects of immune responses and other side effects.

Keywords – immune response, anti-hyperglycemic effect, *Cordyceps militaris*, peritoneal macrophages, cordycepin, anti-diabetes

Introduction

Cordyceps sinensis, known as "Dong Chong Xia Cao" (summer-plant, winter-worm), is one of the most valued Chinese herbal medicine. Wild *Cordyceps sinensis* is rare and it is very difficult to culture in artificially. Chinese scientists developed fermentable strain Cs-4. Its various pharmacological activities, oxygen-free radical scavenging, antisenescence, endocrine, hypolipidemic, antiathrosclerotic, sexual function-restorative, glucose metabolism, the respiratory, hepatic, cardiovascular, immunological, anticancer and effect on kidney disease are well summarized by Zhu *et al.* (1998). On the other hand, *Cordyceps militaris* is another genus of cordyceps, which is widely used in China and Korea. Cordycepin (3'-deoxyadenosine) has been considered as one of the effective components of *Cordyceps sinensis* (Chen and Chu, 1996; Zhu *et al.*, 1998). More earlier, a product from *Cordyceps militaris* was isolated which inhibited growth of *Bacillus subtilis* (Chunningham *et al.*, 1951; Bentley *et al.*, 1951). This compound was later identified as cordycepin (Kaczka, *et al.*, 1964). Since the structure was verified, various biological effects of cordycepin have been studied e.g. anti-fungal activity (Sugar and McCaffrey, 1988), anti-malarial activity (Trigg *et al.*, 1971), anti-herpes activity (Julian-Ortiz *et al.*, 1999), anti-tumorigenic activity on some cell lines (Deitch and Sawicki, 1979), stimulating effect on interleukin-10 production as a immunomodulator (Xiaoxia *et al.*, 2002), and effect of polyadenylation inhibition (Ioannidis *et al.*, 1999). About

*Author for correspondence
Fax: 82-2-948-5370, E-mail: kimkj@syu.ac.kr

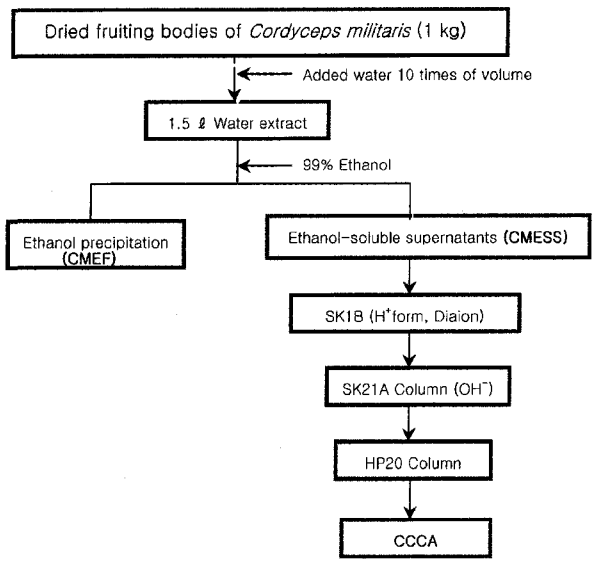
anti-leukemic activity, when protected against ADA deamination, cordycepin is especially cytotoxic for TdT-positive leukemia cells (Koc *et al.*, 1996). Similar results were observed by Kodama *et al.* (2000). In their report, resting cells may be relatively resistant to the toxic effect of cordycepin, where as activated dividing cells are more vulnerable to cordycepin mediated toxicity during possible anti-leukemic therapy. In respect to the anti-diabetic effect, CS-F30, a purified polysaccharide (molecular weight 45,000) isolated from a fermented mycelia product of *Cordyceps sinensis*, exhibited a potent hypoglycemic activity after oral administration in mice (Kiho *et al.*, 1993). By injection (intraperitoneal or intravenous), CS-F30 caused more dramatic hypoglycemic effect: plasma glucose was reduced in normal and diabetic mice (Kiho *et al.*, 1996). CS-F30 increased the activity of hepatic glucokinase, hexokinase, and glucose-6-phosphate dehydrogenase. In this research, We have isolated low molecular weight fractions of *Cordyceps militaris*, CCCA and CMESS, that have a trehalase and α-glucosidase inhibitory activity *in vitro* (data not shown) and evaluated hypoglycemic effect of these compounds on mice and followed their immune response after treatment.

Experimental

Instruments and reagents – Analytical HPLC system (Gilson), Centrifuge (5810R, Eppendorf, Germany), ELISA reader (Emax, Molecular Devices, U.S.A.), CO_2 incubator (Forma Scientific, Inc., Marietta, U.S.A.) and amino acid analyzer (L-8800 AAA System, Hitachi, Japan) were used. Streptozotocin, lipopolysaccharide (LPS: *Escherichia coli* 0111:B4, Con A (concanavalin A), XTT (sodium 3-[1-(phenylaminocarbonyl)-3,4-terazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) reagent, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chem. Co. (St. Louis, MO. USA). DMEM (Dulbecco's Modified Eagle Medium), FBS (fetal bovine serum), Penicillin/Streptomycin were purchased from Gibco (BRL, Grand Island). And sGOT, sGPT kit was purchased from Yeongdong pharmaceutical Co. (Seoul, Korea). All other chemicals and reagents were analytical grade.

Preparation of CCCA and CMESS from *Cordyceps militaris* – The cultured fruit bodies of *Cordyceps militaris* were supplied by CM Biotec, Kangnung, Korea and identified by professor Jae-Mo Sung (department of agricultural biology of Kangwon national university). The dried cultured fruit bodies were extracted with the distilled deionized water (DDW) for 6 hrs. Water extract of cultured fruit bodies was concentrated with a vacuum evaporator at 70°C, and it was diluted three times with DDW. And about 8 times of its volume of 99% ethanol was added to the water extract. The supernatant was decanted and concentrated at 55°C. The ethanol precipitate (CMEP) was discarded. The concentrate of ethanol-soluble supernatant was diluted with water and lyophilized to CMESS. Diluted CMESS was passed through the strong cation exchange resin SK1B column (Diaion, H^+) and 3 times of bed volume of 4% HCl was eluted, the eluate was discarded and subsequently 4% NH_4OH was eluted. The eluate of 4% NH_4OH was got together checking the presence of cordycepin in its concentrate by TLC ($CHCl_3$:MeOH:H_2O=65:35:10, anisaldehyde reagents). The concentrate was diluted again with DDW, passed through the anion exchange resin column (Diaion, OH^-). The eluate was concentrated again to adequate volume, passed through the synthetic adsorbents HP20 column and DDW was eluted with 3 times of bed volume of DDW, the eluate was discarded. For the last time, more than 3 times of bed volume of 5% propyl alcohol was eluted and the eluate was concentrated, diluted again with adequate volume of DDW, lyophilized to CCCA (crude cordycepin containing adenosine) (Scheme 1).

Animals – The CrjBgi:CD-1 male mice were purchased from Bio Genomics in Korea. The experiments were carried out in a pathogen-free barrier zone at Sahmyook university in accordance with the procedure outlined in the guide for the care and use of laboratory animals and were raised in the animal facility of our laboratory from 4 weeks to age 5 weeks for acclimatization. The mice were housed with free access to a standard diet (Samyang Co., Korea) and

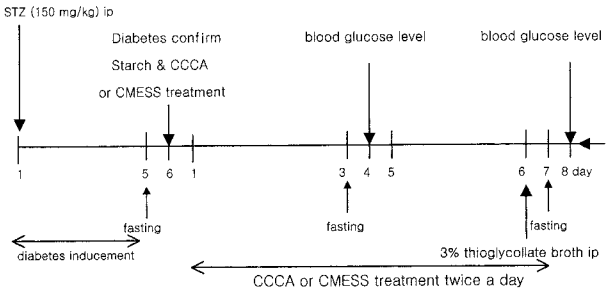


Scheme 1. Extractions and fractionations of *Cordyceps militaris*.

drinking water under 12 hrs cycles of alternating light and dark. Temperature (23±2°C) and humidity (55±5%) were artificially adjusted. Streptozotocin (STZ, 150 mg/kg, Sigma St. Louis, MO. USA) dissolved in 0.01 M citrate buffer (pH 4.5) was injected to the 5 weeks age mice for the diabetic group intraperitoneally and the saline alone was given for the control group. Amount of feed and water consumed was checked at every 2 days. On the 6th day from the injection, after the 12 hrs fasting, the blood samples were obtained from the retro-orbital venous plexus with a heparinized capillary tube (Chase Scientific Glass Inc. USA) and were centrifuged at 4°C, 3000 rpm for 10 min. Glucose level in the supernatant plasma was determined to confirm the induction of diabetic mice by glucose enzymatic method (Asan Pharm. Co. LTD. Korea, licensed by Nissui Pharm. Co. LTD.). The mice with blood glucose levels greater than or equal to 200 mg/dl were regarded as diabetic. These STZ-induced hyperglycemic mice were used as the STZ-control and test group. We weighed the body weight of the control (normal) and STZ-induced diabetic mice and calculated amount of water and food consumed on the same day.

Administration of CCCA, CMESS, and cordycepin – The STZ-induced diabetic mice were divided into 7 groups each (n=6); control group (normal mice), STZ-control group, acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), standard cordycepin (0.2 mg/kg) and standard tryptophan (1 mg/kg) group. After 12 hrs fasting blood glucose levels were obtained by the above method and then the STZ-control group was administered with the starch (2 g/kg) only, other test groups were administered with the starch (2 g/kg) and samples in above dose. Blood glucose level was measured after 60 min. After the starch-loading test, same amount of samples were administered continually two times a day. The sterilized water was administered in the STZ-control. After 12 hrs fasting the blood glucose level was measured on the 4th and 8th day from the first sample administration day. The thioglycollate broth (3%, 2 ml) was injected to the all groups on the 6th day for collecting macrophages (Scheme 2).

Proliferation of peritoneal macrophages and nitric oxide assay – On the 8th day, the body weights were weighed, after collecting blood from the retro-orbital venous plexus of each group with a heparinized capillary tube, 10 ml of cold PBS was injected i.p. and collected again from the abdominal cavity after rubbing the animal abdominal region for 2 min, another 10 ml of PBS was injected and the peritoneal exudates were gathered in the same way. The collected peritoneal exudates containing macrophages were centrifuged at 4°C, 1200 rpm, for 10 min. The supernatant



Scheme 2. Experiment protocol.

was removed and the cell suspension containing red blood cells was treated with 2 ml of ACE (8 g NH_4Cl, 1 g Na_2EDTA, 0.1 g KH_2PO_4, pH 7.4) lysing buffer for 5 min, added PBS and the supernatant was removed after centrifugation. The macrophages was moved on the petri-dish with a 10 ml of culture Dulbecco's Modified Eagle Medium (DMEM) with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% fetal bovine serum and cultured in the CO_2 incubator for 2 hrs (at 37°C, 5.5% CO_2). The macrophages adhered on the bottom of the petridish were collected and adjusted to 2×10^6cells/ml with a complete media (DMEM-10). The both macrophages solutions whether treated with LPS (25 ng/ml) or not, 200 μl were transferred to the every well in 96 well plate and incubated in CO_2 incubator for 30 hrs (at 37°C, 5.5% CO_2) and then 50 μl of MTT reagent (2 mg/ml) was added to the every well, incubated for 4 hrs and the proliferation of macrophages were evaluated by ELISA reader at 540 nm. The supernatant of macrophages solution which was incubated for 30 hrs, 100 μl was transferred to the new 96 well plate, 100 μl of Griess reagents was treated to the every well for 10 min in room temperature and amount of NO was evaluated by ELISA reader at 540 nm.

Preparation and proliferation of splenocytes – After collecting the macrophages on the 8th day, the mice were sacrificed by cervical dislocation, spleen, liver, kidney, muscles were weighed, all the organs were stored in the freezer at −70°C except spleen. The spleen was transferred to the petridish with 10 ml of PBS, teased with micro slide, centrifuged for 10 min at 4°C, 1200 rpm, the supernatants were removed. The cell pellet were loosened by agitation, added 3 ml of ACE lysing buffer, standard for 10 min, added PBS and centrifuged. The supernatants were removed, PBS 10 ml was added to the cell pellet and then amount of splenocytes were adjusted to 1×10^6cells/ml with DMEM-10 media. The splenocytes of each group treated with lipopolysaccharide (LPS, 10 μg/ml, *Escherichia coli* 0111:B4), Con A (concanavalin A, 4 μg/ml) and not treated, 200 μl were transferred to the each well of the new 96 well plate

(2×10^5cell/200 μl) and incubated for 3 days in the CO_2 incubator (at 37°C, 5.5% CO_2). XTT reagents (1 mg/ml, 50 μl) were added to the 96 well plate, proliferation of splenocytes was evaluated after 3 hrs incubation by ELISA reader at 490 nm.

Measurement of serum GOT and GPT – The levels of sGOT (serum glutamic oxaloacetic transaminase) and sGPT (serum glutamic pyruvic transaminase) of the blood samples collected from retro-orbital venous plexus of each group with a heparinized capillary tube were measured by the Reitman-Frankel's method (1957).

Statistical analysis – Data are expressed as the mean ± SD. Comparisons were analyzed by using Students *t*-test for unpaired data. Differences were considered significant when *P* values equal or less than 0.05 were obtained. The software used was the Statistical Package for Social Sciences.

Results

Cordycepin (C) and adenosine (A) in CCCA –The amounts of cordycepin and adenosine in CCCA analyzed by HPLC were 164 mg/g (16.4%) and 253 mg/g (25.3%), respectively (Fig. 1a). Also, PH-TEST showed that CCCA contains tryptophan (34.8 mg/g) and phenylalanine (33.43 mg/g) (Fig. 1b). The contents of cordycepin and adenosine in CMESS were 4.576 mg/g (0.46%) and 7.261 mg/g (0.73%). The amounts of cordycepin and adenosine in CCCA were about forty fold to CMESS. CCCA, CMESS, standard cordycepin, and tryptophan (Sigma, St. Louis, MO. USA) were used in this research. Concentration of CCCA and CMESS were administrated by 4 mg/kg and 50 mg/kg respectively. The amount of cordycepin and adenosine treated in CCCA were about three times higher than that of CMESS *in vivo* test (Table 1).

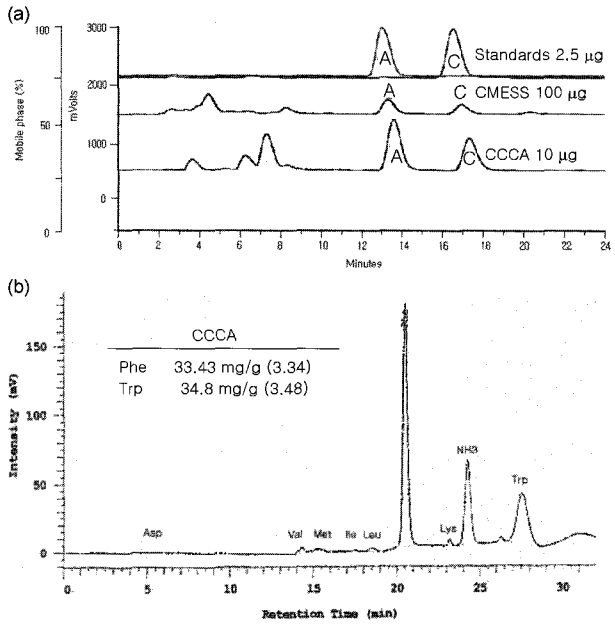


Fig. 1. Typical HPLC chromatograms (**1a**) and amino acids analysis (**1b**) of cordycepin (**C**), adenosine (**A**), and other amino acids in CCCA and CMESS.

Changes in body weight, water, and food intakes in STZ-induced diabetic mice after inducing diabetes – The body weight and the water intakes of STZinduced diabetic mice were decreased 11% and 11.6% respectively as compared with the normal mice. On the contrary water intakes increased two fold to the normal mice. These facts indicate that diabetes was successfully induced (Table 2, $p<0.0001$).

Anti-diabetic effect of CCCA and CMESS in STZ-induced diabetic mice before and after administration of starch (2 g/kg) – As shown in Table 3, the amounts of CCCA, CMESS, and cordycepin was described. In the starch over loading test, after 60 min of administration acarbose (10 mg/kg) and CMESS (50 mg/kg) showed significant decrease ($p<0.05$, 37.8 and 34.7% respectively) of blood glucose levels in the STZ-induced diabetic mice (Table 4).

Table 1. Administration amounts of cordycepin and adenosine in CCCA and CMESS

Purification steps	Contents	
	Cordycepin (%)	Adenosine(%)
CCCA	164.5 mg/g (16.4)	253.2 mg/g (25.3)
CMESS	4.576 mg/g (0.46)	7.261 mg/g (0.73)
CCCA (4 mg/kg/mouse)	658 μg/4 mg	1012.8 μg/4 mg
CMESS (50 mg/kg/mouse)	228.8 μg/50 mg	363.1 μg/50 mg

Table 2. Changes in body weight, water, and food intakes in normal and STZ-induced diabetic mice during one week

	Body weight (g)	Water intake (ml/mouse/day)	Food intake (g/mouse/day)
Control	31.72±0.23*	7.71±0.21*	6.89±0.36*
STZ-induced mice	28.22±2.53	14.54±3.26	6.09±0.15

Water and food intakes were measured every 2 day.
Data are represented as mean±S.D. (control n=6, STZ-induced mice n=45).
*Significantly different from control (normal mice) at $P< 0.0001$.

Table 3. Treatment protocol of CCCA, CMESS, and cordycepin with starch (2 g/kg)

Groups	Treatment
Control	Distilled water
STZ-control	Starch (2 g/kg)
Acarbose	Starch + 10 mg/kg
CCCA	Starch + 4 mg/kg
CMESS	Starch + 50 mg/kg
Cordycepin	Starch + 0.2 mg/kg
Tryptophan	Starch + 1 mg/kg

All groups were classified according to 6 mice per each group.

Table 4. Anti-diabetic effects of CCCA and CMESS in STZ-induced diabetic mice before and after administration of starch (2 g/kg)

Group	Blood glucose level (mg/dl)		
	Before	After	After-Before
STZ-control	461.9±73.2	637.5±26.9*	175.6
Acarbose	466.8±62.8	401.2±76.2*	-65.6
CCCA	466.5±69.3	555.1±51.4	88.6
CMESS	471.5±65.9	424.9±50.7*	-46.6
Cordycepin	472.2±60.5	593.8±62.3	121.6
Tryptophan	466.1±56.0	516.6±94.9	50.5

Each sample was administered with a starch (2 g/kg) in oral after overnight fasting. Blood glucose level was measured before and 60 min after administration. STZ-control: STZ-induced diabetic mice, Acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), Cordycepin (0.2 mg/kg), Tryptophan (1 mg/kg).
Control group was administered with distilled water.
Data are represented as mean S.D. of 6 mice per each group.
*Significantly different from control at P<0.05.

Table 5. Duration effects of anti-diabetic activities of CCCA, CMESS, and cordycepin in STZ-induced diabetic mice after 3 and 7 days administration

STZ-induced Group	Blood glucose level (mg/dl)	
	3 days	7 days
STZ-control	566.17±18.44	656.77±26.83
acarbose	355.42±76.14*	410.19±137.98*
CCCA	538.12±33.63	578.33±19.35
CMESS	365.44±11.81*	348.72±101.94*
Cordycepin	538.47±40.68	338.95±39.89
Tryptophan	479.16±71.59	452.97±98.99

STZ-control: STZ-induced diabetic mice, Acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), Cordycepin (0.2 mg/kg), Tryptophan (1 mg/kg).
All samples were administrated in oral twice a day.
Control group was administered with distilled water.
Data are represented as mean S.D. of 6 mice per each group.
*Significantly different from control at P < 0.05.

Duration effects of anti-diabetic activities of CCCA, CMESS, and cordycepin in STZ-induced diabetic mice after 3 and 7 days administration – After 3 days administration, acarbose and CMESS reduced blood glucose level by 37.2 and 35.5% respectively. CCCA and cordycepin showed no significant effects. Acarbose, CMESS, and cordycepin exhibited potent hypoglycemic activities after 7 days administration (inhibition ratio, 37.5, 46.9 and 48.4% respectively). CCCA that has three fold amounts of cordycepin showed no hypoglycemic effect. And tryptophan showed a little hypoglycemic effects with no significances (Table 5).

Changes of various organ weights after CCCA, CMESS, and cordycepin administration – Weight of muscle, liver, spleen, and kidney were observed to see its possibility of safe usage of the alternative drug for the diabetes. In our experiments, comparing STZ-induced diabetic mice with cordycepin (0.2 mg/kg) and CMESS (50 mg/kg) have no significance. But STZ-control, acarbose (10 mg/kg) and CCCA (4 mg/kg) showed significant differences from the control mice (P<0.001, P<0.005 and P<0.05 respectively, listed in Table 6). These results say that CMESS and cordycepin can be the useful alternative drugs.

Effects on the NO production in LPS-treated peritoneal macrophages derived from STZ induced diabetic mice administered CCCA, CMESS, and cordycepin – LPS (25 ng/ml) treated peritoneal macrophages derived from STZ-induced diabetic mice administered cordycepin (0.2 mg/kg) for 7 days enhanced the NO production (98.37 μM). The levels of NO production in this group were more than two fold to LPS-treated STZ-control (39.93 μM). While the LPS treated macrophages derived from the STZ induced diabetic mice administered CMESS (50 mg/kg) reduced the NO production (7.16 μM) less than the LPS-untreated normal control group (8.31 μM). CCCA (4 mg/kg) that has high contents of cordycepin (656 μg/4 mg) did not enhance the NO production (Fig. 2).

Table 6. Measurement of muscle, liver, and kidney weight in normal and STZ -induced diabetic mice

Group	Muscle/body weight ($X10^{-2}$)	Liver/body weight ($X10^{-2}$)	Spleen/body weight ($X10^{-3}$)	Kidney/body weight ($X10^{-2}$)
Control	7.57±0.73	4.75±0.22	4.82±0.36	1.68±0.07
STZ-control	8.03±0.68	7.59±0.88**	5.73±0.13	2.32±0.11*
Acarbose	8.50±0.59	6.47±0.60	6.38±0.11***	2.19±0.20**
CCCA	8.67±0.79	7.09±0.65***	6.33±0.21***	2.10±0.09***
CMESS	6.69±2.08	5.46±0.24	5.35±0.60	1.85±0.06
Cordycepin	8.09±0.58	5.37±0.67	5.40±0.21	1.77±0.10
Tryptophan	8.32±0.54	5.14±0.41	5.79±0.44	1.90±0.06

All samples (same dose with Table 4) were administrated twice a day. Animals were sacrificed on the 8th day by decapitation since first administration.
Data are represented as mean±S.D. of 6 mice per each group.
*Significantly different from control at P < 0.001.
**Significantly different from control at P < 0.005.
***Significantly different from control at P < 0.05.

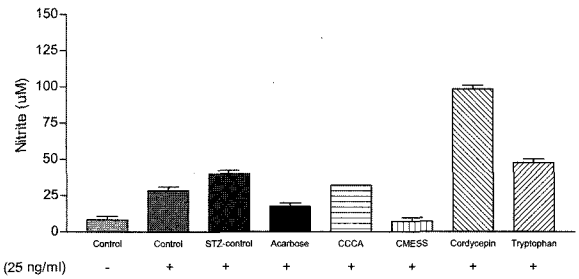


Fig. 2. Nitric oxide productions in LPS (25 ng/ml) treated peritoneal macrophages derived from STZ-induced diabetic mice administered with CCCA, CMESS, and cordycepin. Macrophages were collected on the 8th day since first administration of samples. Dose of administered samples: Acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), Cordycepin (0.2 mg/kg), Tryptophan (1 mg/kg). All samples were administered two times a day.

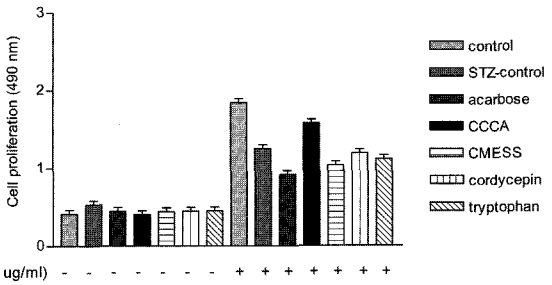


Fig. 3. Effect on the proliferation of LPS (10 μg/ml)-treated splenocytes derived from STZ-induced diabetic mice administered with CCCA, CMESS, and cordycepin.
Control: Normal mice, STZ-control: STZ-induced diabetic mice, Acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), Cordycepin (0.2 mg/kg), Tryptophan (1 mg/kg)
All samples were administrated twice a day during one week.

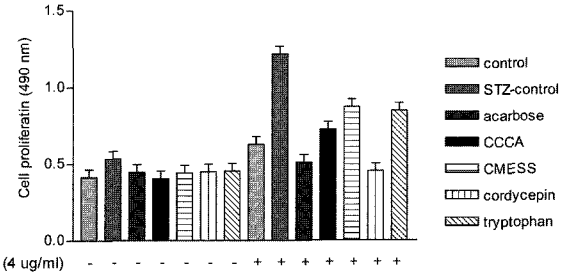


Fig. 4. Effect on the proliferation of Con A (4 μg/ml)-treated splenocytes derived from STZ-induced diabetic mice administered with CCCA, CMESS, and cordycepin.
Control: Normal mice, STZ-control: STZ-induced diabetic mice, Acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), Cordycepin (0.2 mg/kg), Tryptophan (1 mg/kg).
All samples were administrated twice a day during one week.

Effect on proliferation of LPS-treated peritoneal macrophages derived from STZ-induced diabetic mice administered CCCA, CMESS, and cordycepin – There were no significant differences between proliferation of macrophages before and after treating with LPS in all group. CCCA showed higher viability than other groups. On the other hand CMESS exhibited lower viability than normal mice as control and STZ-control group (data not shown).

Effects on proliferation of splenocytes derived from STZ-induced diabetic mice administered with either CCCA, CMESS, or cordycepin – In observation of the splenocytes derived from the sample administered STZ-induced diabetic mice, there are no distinct differences in both LPS and Con A untreated groups, while CCCA administered group exhibited increased proliferation than STZ-control, decreased than control (normal) group and other groups showed lower proliferation than STZ-control in LPS treated STZ-induced diabetic groups (Fig. 3). CCCA, CMESS, and tryptophan showed increased proliferation than control group, and decreased than STZ-control group, acarbose, and cordycepin groups exhibited decreased proliferation than the control group in Con A treated splenocytes from STZ-induced diabetic group (Fig. 4). These facts indicate that cordycepin and CMESS play an important role in immune responses like immunomodulatory, immunosuppressive activities.

Effects of CCCA, CMESS, and cordycepin on levels of sGOT and sGPT in STZ-induced diabetic mice – The levels of sGOT and sGPT for evaluating the influences on liver metabolism were carried out by Reitman-Frankels method. The levels of sGOT (76 unit) and sGPT (42 unit) of STZ-control were higher than control (both 24 unit). In most case, STZ-induced diabetes has liver damage (Park *et al.*, 1992). Standard cordycepin and tryptophan exhibited increased sGOT levels than control group. Acarbose and CCCA showed lower levels of the enzyme activity than control. There were no significant differences in all groups except cordycepin in the levels of sGPT. In these results cordycepin alone increased the levels of both enzymes, but not in CCCA and CMESS which containing cordycepin (Fig. 5).

Discussion

Our major finding of this research is observation that cordycepin, already known as a polyadenylation inhibitor, has a potent anti-diabetic activity *in vivo*. We observed increasing of NO production in the LPS treated peritoneal macrophages and decreasing of T-lymphocyte proliferation in the Con A treated splenocytes, derived from STZ-induced diabetic mice administered cordycepin (0.2 mg/kg, for 7 days). Another finding is that CMESS, containing cordycepin (0.23 mg/50 mg), also has a potent anti-diabetic effect *in vivo*, decreased NO production in LPS-treated and untreated peritoneal macrophages.

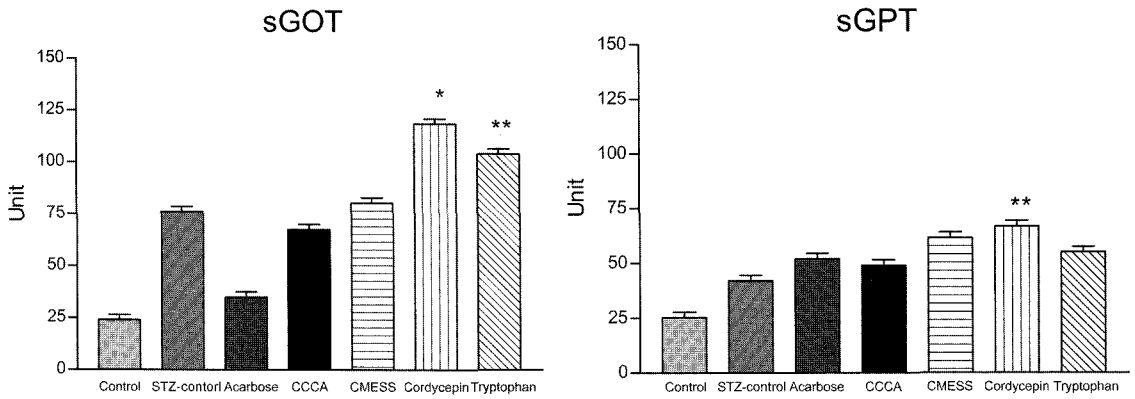


Fig. 5. Effects of CCCA, CMESS, and cordycepin on the blood serum glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT) levels in mice. Control: Normal mice, STZ-control: STZ-induced diabetic mice, Acarbose (10 mg/kg), CCCA (4 mg/kg), CMESS (50 mg/kg), Cordycepin (0.2 mg/kg), Tryptophan (1 mg/kg).
All samples were administrated twice a day.
*Significantly different from control at $P < 0.01$.
**Significantly different from control at $P < 0.05$.

In order to investigate the effect of CCCA, CMESS, and cordycepin on lymphocyte proliferation, the splenocytes were incubated with either LPS, mitogen for B-lymphocytes, or Con A, mitogen for T lymphocytes. T-lymphocytes proliferation in Con A treated STZ-control was increased. But the proliferation of T-lymphocytes treated with cordycepin (0.2 mg/kg) was significantly lower than STZ-control in the presence of Con A.

Insulin-dependent diabetes mellitus (IDDM) is a progressive autoimmune disease in genetically susceptible individuals, resulting in inflammatory damage to pancreatic β-cell islets (Lukic *et al.*, 1991). Genetic and environmental factors are the key contributors to the susceptibility of the disease. A susceptible person develops insulitis and eventually the disease by an autoimmune response thought to be mediated by T-lymphocytes that are reactive to islet β-cell auto-antigen. These $CD4^+/CD8^+$ T-lymphocytes directly cause β-cell damage or through indirect mechanisms, such as by producing cytokines and free radicals and inducing β-cell programmed cell death or apoptosis (Anjli *et al.*, 1999).

Although further investigation is needed, we hypothesize that anti-diabetic effect of cordycepin is due to decreasing T-lymphocytes, leading the pancreatic β-cell to restore, by cytotoxycity, acting as a immunomodulator or apoptosis induced by NO production, a powerful proliferation inhibitor of T-lymphocyte. The hypothesis in this animal model is enough to explain well the reason why cordycepin failed to reduce blood glucose level on the 3rd day since first administration but dropped the level dramatically on the 7th day. It is thought to be that first 3 days was a recovering time from insulitis by STZ, since then pancreatic β-cell was able to release insulin. It is uncertain whether increasing of NO production of LPS stimulated macrophages from cordycepin treated STZ-mice is related in NO synthesis or not.

NO plays an important role in glucose metabolism like inducing insulin release (Nakata and Yada, 2003), stimulation of glucose uptake to skeletal muscle and adipose tissue (Roy *et al.*, 1998; Balon and Nadler, 1997). However, the involvement of NO in glucose regulation remains unclear. NO mediated post neonatal β-cell apoptosis occurs in *vivo* in calmodulin-transgenic mice. Apoptosis of β-cell was accelerated by sulfonylurea, tolbutamide, which overproduced calmodulin. Ca^{2+} dependent apoptosis of pancreatic β-cell cause by calmodulin over expression was prevented by NOS inhibitor (Yu *et al.*, 2002). Nitric oxide synthase (NOS) inhibitor, L-NAME, significantly reduced 2-deoxyglucose-induced hyperglycemia in rats (Sugimoto, *et al.*, 1997). The abnormalities in urinary hemodynamics (diuresis, proteinuria and kallikrein excretion) detected in STZ-induced diabetic model (Type I) were reversed by the L-NAME (Catanzaro *et al.*, 1994). It prevent inflammatory mechanism of diabetes type I, the vascular dysfunction and structural changes occurring early after the onset of diabetes. Indeed, activities of NO on diabetes have two faces, beneficial on one hand and harmful on the other hand depending on the biological or experimental set up conditions. Of course more research is needed, it seems to be that anti-diabetic effect of CMESS was due to decreasing NO synthesis (Fig. 2) by nitric oxide synthase inhibitors it contains. In spite of its high content of cordycepin, CCCA administered group could not reduced blood glucose level. This fact indicates that anti-diabetic activity of cordycepin is not a dose dependent manner or CCCA may have a compound of opposite action. Cordycepin enhanced the activities of sGOT and sGPT. So lower

concentration of cordycepin should be used for diabetes. All things considered, we concluded that cordycepin and CMESS may be useful regulating tools of hyperglycemia as an immunomodulator or immunosuppresant in type I diabetes, which is progressive or induced from anti-diabetic drug like sulfonylureas.

References

Anjli, K., and Noel, K., Maclaren, Autoimmunity and diabetes. *J. Clin. Endocrinol.* **84**, 4371-4378 (1999).

Balon, T. W., and Nadler, J. L., Evidence that nitric oxide increase glucose transport in skeletal muscle. *J. Appl. Physiol.* **82**, 359-363 (1997).

Bentley, H. R., Cunningham, K. G., and Spring, F. S., Cordycepin a metabolic product from cultures of *Cordyceps militaris* link part II. The structure of cordycepin. *J. Chem. Soc.* 2301-2305 (1951).

Catanzaro, O. L., Marina-Prendes, M. G., Hope, S. I., Zuccollo, A., and Dominguez, A., Streptozotocin-induced hyperglycemia is decreased by nitric oxide inhibition. *Braz. J. Med. Biol. Res.* **27**, 2043-2047 (1994).

Chen, S. Z., and Chu, J. Z., NMR and IR studies on the characterization of cordycepin and 2-deoxyadenosine. *Zhongguo Kangshengsu Zazhi* **21**, 9-12 (1996).

Cunningham, K. G., Hutchinson, S. A., Manson, W., and Spring, F. S., Cordycepin. A metabolic product from cultures *Cordyceps militaris* Link. Part I. Isolation and characterization. *J. Chem. Soc.* 2299-3000 (1951).

Deitch, A. D., and Sawicki, S. G., Effects of cordycepin on microtubules of cultured mammalian cells. *Exp. Cell Res.* **118**, 1-13 (1979).

Ioannidis, P., Courtis, N., Havredaki, M., Michailakis, E., Tsiapalis, C. M., and Trangas, T., The polyadenylation inhibitor cordycepin (3'dA) causes a decline in c-MYC mRNA levels without affecting c-MYC protein levels. *Oncogene* **18**,117-125 (1999).

Julian-Ortiz, J. V., Galvez, J., Munoz-Collado, C., Garcia-Domenech, R., and Gimeno-Cardona, C., Virtual combinatorial syntheses and computational screening of new potential anti-herpes compounds. *J. Med. Chem.* **42**, 3308-3314 (1999).

Kaczka, E. A., Trenner, N. R., Arison, B., Walker, R. W., and Folkers, K. Identification of cordycepin, a metabolite of *Cordyceps militaris*, as 3'-deoxyadenosine. *Biochim. Biophys. Res. Commun.* **14**, 456-457 (1964).

Kiho, T., Hui, J., Yamane, A., and Ukai, S., Polysaccharides in fungi. XXXII. Hypoglycemic activity and chemical properties of a polysaccharide from the cultural mycelium of *Cordyceps sinensis*. *Biol. Pharm. Bull.* **16**, 1291-1293 (1993).

Kiho, T., Yamane, A., Hui, J., Usui, S., and Ukai, S., Polysaccharides in fungi. XXXVI. Hypoglycemic activity of a polysaccharide (CS-F30) from the cultural mycelium of *Cordyceps sinensis* and its effect on glucose metabolism in mouse liver. *Biol. Pharm. Bull.* **19**, 294-296 (1996).

Koc, Y., Urbano, A. G., Sweeney, E. B., and McCaffrey, R., Induction of apoptosis by cordycepin in ADA-inhibited TdT-positive leukemia cells. *Leukemia* **10**, 1019-1024 (1996).

Kodama, E. N., McCaffrey, R. P., Yusa, K., and Mitsuya, H., Antileukemic activity and mechanism of action of cordycepin against terminal deoxynucleotidyl transferase-positive (TdT+) leukemic cells. *Biochem. Pharm.* **59**, 273-281 (2000).

Lukic, M. L., Stosic-Grujicic, Ostojic, N., Chan, N. L., and Liew, F. Y., Inhibition of nitric oxide generation affects the induction of diabetes by streptozotocin in mice. *Biochem. Biophys. Res. Commun.* **178**, 913-920 (1991).

Nakata, M., and Yada, T., Nitric oxide-mediated insulin secretion in response to citrulline in islet beta-cells, pancreas. *Endocrinology* **27**, 209-213 (2003).

Park, K. S., Chong, J. C., Moon, C. K., and Chung, J. H., Toxicity of streptozotocin in isolated rat hepatocytes. *Yakhak Hoeji* **36**, 80-86 (1992).

Roy, D., Perreault, M., and Marette, A., Insulin stimulation of glucose uptake in skeletal muscles and adipose tissues *in vivo* is NO dependent. *Am. J. Physiol.* **274**, E692-E699 (1998).

Sugar, A. M., and McCaffrey, R. P., Antifungal activity of 3'-deoxyadenosine (cordycepin). *Agents Chemother.* **42**, 1424-1427 (1998).

Sugimoto, Y., Yamada, J., Yoshikawa, T., and Horisaka, K., Inhibitory effects of a nitric oxide synthase inhibitor, N(G)-nitro-L-arginine methyl ester (L-NAME), on 2-deoxy-D-glucose-induced hyperglycemia in rats. *Biol. Pharm. Bull.* **20**, 1307-1309 (1997).

Trigg, P. I., Gutteridge, W. E., and Williamson, J., The effects of cordycepin on malaria parasites. *Trans. R. Soc. Trop. Med. Hyg.* **65**, 514-520 (1971).

Xiaoxia, Z., Claudius, U. M., Peter, S., and Fred, Z., Effect of cordycepin on interleukin-10 production of human peripheral blood mononuclear cells. *Eur. J. Pharmacol.* **453**, 309-317 (2002).

Yu, W., Niwa, T., Miura, Y., Horio, F., Teradaira, S., Ribar, T. J., Means, A. R., Hasegawa, Y., Senda, T., and Niki, I., Calmodulin overexpression causes Ca^{2+} dependent apoptosis of pancreatic beta cells, which can be prevented by inhibition of nitric oxide synthase. *Lab. Invest.* **82**, 1229-1239 (2002).

Zhu, J. S., Halpern, G. M., and Jones, K., The scientific rediscovery of an ancient Chinese herbal medicine: *Cordyceps sinensis*: part I. *J. Altern. Complement. Med.* **4**, 289-303 (1998).

(Accepted December 10, 2003)