The effect of cordycepin on brain oxidative stress and protein expression in streptozotocin-induced diabetic mice

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ABSTRACT. Diabetes mellitus (DM) is characterized by metabolic disorders and psychological deficits, including cognitive decline. Here, we investigated the effect of cordycepin on oxidative stress and protein expression in the brains of diabetic mice. Twenty-four mice were divided into four groups, one comprising untreated healthy mice (N); one comprising healthy mice treated with cordycepin (24 mg/kg body weight) (N+Cor); one comprising untreated DM mice; and one comprising DM mice treated with cordycepin (24 mg/kg body weight) (DM+Cor). After 14 days of treatment, cognitive behavior was assessed using the novel object recognition (NOR) test. The brain levels of oxidative stress markers (glutathione, catalase, and superoxide dismutase) were examined using the respective detection kits. Protein expression in brain tissues was assessed by liquid chromatography with tandem mass spectrometry (LC–MS/MS); the functions of the identified proteins were annotated by PANTHER, while major protein–protein interactions were assessed using STITCH. We found that cordycepin treatment significantly decreased body weight and food and water intake in the DM+Cor group compared with that in the DM group; however, no differences in blood glucose levels were found between the two groups. Cordycepin treatment significantly reversed cognitive decline in diabetic mice in the NOR test and ameliorated antioxidant defenses. Additionally, we identified ULK1 isoform 2, a protein associated with cognitive function *via* the activated AMPK and autophagic pathways, as being uniquely expressed in the DM+Cor group. Our findings provide novel insights into the cellular mechanisms underlying how cordycepin improves cognitive decline in diabetic mice.

KEY WORDS: brain protein, cognitive function, cordycepin, diabetes mellitus, free radical

Diabetes mellitus (DM) is a metabolic disease in which the body does not produce enough insulin or cannot use it effectively, leading to a high blood glucose concentration [3]. DM results in tissue and organ dysfunction, leading to several systemic complications including heart disease, retinopathy, nephropathy, and impaired reproductive and brain function [67]. Diabetic-associated cognitive decline (DACD) is characterized by reduced performance in several cognitive domains, a slowing of mental speed, and diminished flexibility. Although patients present with mild to moderate symptoms, DACD can nonetheless significantly hamper daily functioning and increase the risk for dementia, Alzheimer's disease, and affective disorders [16, 44, 45]. Although the mechanism underlying the pathogenesis of DACD has not been fully elucidated, existing evidence indicates that cognitive decline in DM patients may be a multifactorial process [59], with brain oxidative stress playing a significant role [30, 33, 34, 71]. Oxidative stress occurs when there is an imbalance between excess reactive oxygen species (ROS) production and cellular antioxidant capacity [73]. The brain is especially sensitive to oxidative stress owing to its high oxygen consumption rate, abundant lipid content, and low levels of antioxidant enzymes when compared with other tissues [48]. However, to date, no specific treatment exists for the prevention or treatment of DACD [59].

Cordyceps militaris is a parasite of moth caterpillar larvae used widely in traditional medicine to maintain health and treat several diseases [23]. Cordycepin (3′-deoxyadenosine) is a major bioactive compound derived from *C. militaris,* and has been reported to have multiple biological properties, such as hypoglycemic, antioxidant, anti-inflammatory, and antitumor activities

[4, 27, 58, 79]. Increasing evidence has indicated that cordycepin exerts neuroprotective effects through increasing the level of brain-derived neurotrophic factor (BDNF), the numbers of neuron cells, and activation of autophagy in neuronal cells [9, 43, 69]. Additionally, cordycepin can reportedly reduce postischemic cell death in the hippocampus of rats by inducing membrane hyperpolarization [77]. However, no study to date has investigated the effect of cordycepin on DACD.

Proteomics is a powerful tool widely used to investigate protein expression and identify potential biomarkers or activated proteins [37]. This technique can be used to identify proteins regulated by post-translational modification that cannot be detected using genomic or transcriptomic methods [32]. The aim of the present study was to investigate the effects of cordycepin on protein expression in the brains of streptozotocin (STZ)-induced diabetic mice and clarify the mechanisms underlying its effects on diabetes-associated cognitive impairment.

MATERIALS AND METHODS

Animal care

Twenty-four male C57BL/6Mlac mice (6 weeks old, weighing 25–30 g) were obtained from the National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. The animals were allowed to acclimatize to the laboratory environment for one week before the experiment. The animals were kept under controlled conditions, including a 12:12 hr light: dark cycle, room temperature of $25 \pm 2°C$, and relative humidity of 60–70%, and had free access to water and mouse chow. All the procedures were approved by the Animal Use and Care Committee of Kasetsart University Research and Development Institute, Kasetsart University, Thailand (Approval no. ACKU60-SCI-014).

Induction of diabetes

Type I DM was induced by a single intraperitoneal injection of STZ (200 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in ice-cold citrate buffer (0.1 M, pH 4.5). After 72 hr, the blood glucose level was evaluated using a blood glucose meter (Accu Chek Active, Roche Diagnostic, Mannheim, Germany). Animals with a fasting blood glucose level of ≥250 mg/dl were considered to be diabetic mice and were used for the present study.

Experimental design

According to Ma *et al.* [42], the animals were intraperitoneal administrated cordycepin at dose 24 mg/kg body weight. Hence, mice were randomly divided into 4 groups (*n*=6 per group). Group 1 consisted of healthy control mice (N) who were administered vehicle; Group 2 consisted of healthy mice treated with cordycepin at a dose of 24 mg/kg body weight (N+Cor); Group 3 comprised diabetic mice treated with vehicle (DM); and Group 4 consisted of diabetic mice treated with cordycepin at a dose of 24 mg/kg body weight (DM+Cor). The animals were treated with vehicle or cordycepin (Sigma-Aldrich) by intraperitoneal injection for 14 consecutive days.

Blood glucose level, body weight, food intake, and water intake of the animals

The blood glucose level and body weight of the mice in all the groups were monitored once a week during the experiment. The fasting blood glucose level was measured using a blood glucose meter (Accu-Chek Active). The daily food intake of each mouse was measured by weighing the leftover chow, with food spillage included in the intake measurement.

Novel object recognition test

The novel object recognition (NOR) test, widely used in rodents to assess their ability to recognize a novel object in a familiar environment, was performed according to a previously described method [6] with some modifications. The experiments were carried out in a white plastic box (24 × 32 × 16 cm). Approximately 24 hr before the test, the mice were habituated for 10 min with no objects present. Familiarization was conducted for 5 min by placing individual mice in the open field in which two identical objects (objects A1 and A2; both green spheres) had been positioned in adjacent corners 10 cm away from the walls. After 4 hr, the test trial was conducted by exposing the animals to the familiar object and a novel object (green cube) placed at the same location for 5 min. To control the odor cues, the test box and objects were cleaned with 10% ethanol after each session and each mouse.

During the familiarization and test trial, the time exploring each object was recorded. Object recognition was defined as mice sniffing or touching the object with its nose and/or forepaws within 2 cm or less. The recognition index (RI) was defined as the percentage of time spent exploring the novel object relative to the time spent exploring the familiar and the novel object in the test trial.

Tissue preparation

At the end of the experiment, all the animals were sacrificed by intraperitoneal injection of pentobarbital sodium at a dose of 60 mg/kg. The brain was dissected, rinsed in ice-cold isotonic saline to remove extraneous materials, homogenized in ice-cold phosphate buffer and divided into 2 parts. One part subjected to oxidative stress marker analysis. The other part was diluted with acetone at a 2:1 (*v/v*) ratio and then centrifuged at $10,000 \times g$ for 10 min for proteomic analysis. Protein concentrations were determined by the Bradford assay with bovine serum albumin as the standard.

Oxidative stress markers

Glutathione: Glutathione (GSH) activity was determined according to Lacoste *et al*. [35]. One milliliter of homogenate and 1 ml of 10% trichloroacetic acid (TCA) were centrifuged at 8,960 × *g* for 10 min. Then, 0.5 ml of supernatant was mixed with 2 ml of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and phosphate buffer to a final volume of 3 ml. The absorbance of the supernatant was read at 412 nm. A standard curve was established with various concentrations of GSH (0, 0.013, 0.016, 0.033, 0.067, 0.100, 0.133, and 0.167 mM; Merck, Darmstadt, Germany). The results were expressed as mM of GSH/mg of protein.

Catalase: Catalase (CAT) activity was assayed by the method of Mokhtari-Zaer *et al*. [47] with some modifications. This method is based on the ability of CAT to decompose hydrogen peroxide (H_2O_2) measured at 240 nm. Briefly, the assay mixture consisted of 1.95 ml of potassium phosphate buffer (0.05 M, pH 7.0), 1 ml of hydrogen peroxide (10 mM), and 0.05 ml of brain supernatant in a final volume of 3 ml. The reaction was started by adding H_2O_2 and the reduction in absorption was measured by continuous monitoring by spectrophotometer at 240 nm for 3 min. CAT activity was expressed as units/mg of protein.

Superoxide dismutase: Superoxide dismutase (SOD) activity was determined according to Radovanović *et al*. [55]. SOD activity was assayed using the epinephrine method, which is based on the capacity of SOD to inhibit the autoxidation of adrenaline to adrenochrome. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autoxidation of adrenaline at 26°C and was determined by measuring the absorbance at 480 nm. SOD activity was expressed as units/mg of protein.

Proteomic analysis

Samples from each group were pooled by mixing equal amounts of protein from individual tissue samples. Protein samples were reduced by adding 10 mM dithiothreitol in 10 mM ammonium bicarbonate and alkylated with 30 mM iodoacetamide in 10 mM ammonium bicarbonate. The protein samples were digested with sequencing-grade porcine trypsin (1:20 ratio) for 16 hr at 37°C. The tryptic peptides were dried using a speed vacuum and resuspended in 0.1% formic acid for nano-liquid chromatography–tandem mass spectrometric (nanoLC–MS/MS) analysis.

Liquid chromatography–tandem mass spectrometric analysis and protein identification

The tryptic peptide samples were prepared for injection into an Ultimate3000 Nano/Capillary LC System (Thermo Scientific, Leicestershire, UK) coupled to a HCTUltra LC–MS system (Bruker Daltonics Ltd., Hamburg, Germany) equipped with a nano-captive spray ion source. Briefly, 5 µl of peptide digests were enriched on a µ-Precolumn (300 µm i.d. × 5 mm C18 Pepmap 100, 5 µm, 100 Å) (Thermo Scientific), separated on a 75-µm I.D. × 15 cm PepMap Nanocolumn, and packed with an Acclaim PepMap RSLC C18, 2 µm, 100 Å, nanoViper analytical column (Thermo Scientific). The C18 column was enclosed in a column oven set to 60°C. Solvent A and B containing 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile, respectively, were used. The peptides were eluted using a gradient of 5–55% solvent B at a constant flow rate of 0.30 µl/min for 30 min. Electrospray ionization was carried out at 1.6 kV using the CaptiveSpray source. Nitrogen was used as the drying gas (a flow rate of approximately 50 l/hr). Product ion mass spectra generated by collision-induced dissociation (CID) were obtained using nitrogen as the collision gas. Mass and tandem mass spectra were obtained in positive-ion mode at 2 Hz over the range of 150–2,200 *m/z*. The collision energy was adjusted to 10 eV as a function of the *m/z* value. The LC–MS analysis of each sample was performed in triplicate.

Statistical analysis

Data are expressed as means ± SD and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test using the R project for statistical computing package [54]. A *P*-value <0.05 was considered statistically significant.

RESULTS

Effects of cordycepin on food intake, water intake, body weight, and blood glucose levels

Diabetic mice showed significantly increased food and water intake when compared with normal mice (Table 1). Interestingly, cordycepin treatment resulted in a significant reduction in food and water intake by the diabetic mice compared with that for

Table 1. Effect of cordycepin on food intake, water intake, body weight and blood glucose levels

Parameter	Group			
	N	N+Cor	DM	DM+Cor
Food intake (g/day)	2.71 ± 0.20[a]	2.49 ± 0.16[a]	4.23 ± 0.40[b]	3.47 ± 0.56[c]
Water intake (ml/day)	4.27 ± 1.08[a]	3.31 ± 0.82[a]	20.34 ± 2.98[b]	16.96 ± 3.21[c]
Body weight (g)	24.58 ± 0.73[a]	22.27 ± 1.02[b]	20.41 ± 1.90[b]	18.10 ± 0.51[c]
Blood glucose (mg/dl)	170.83 ± 17.28[a]	148.00 ± 9.101[a]	454.75 ± 196.51[b]	596.00 ± 6.93[b]

All data show as mean ± SD. Value in each row marked different superscript letter differs significantly (*P*<0.05). N, healthy control mice treated with vehicle; N+Cor, healthy mice treated with cordycepin at a dose of 24 mg/kg body weight; DM, diabetic mice treated with vehicle; DM+Cor, diabetic mice treated with cordycepin at a dose of 24 mg/kg body weight.

untreated diabetic mice. In contrast, in normal mice, cordycepin treatment had no effect on food or water intake. Nevertheless, cordycepin administration to normal mice markedly reduced body weight compared with that of controls. Cordycepin treatment did not affect the blood glucose level in diabetic mice.

The effects of cordycepin on cognitive ability

Diabetic mice displayed a significantly lower RI compared with those of the other groups. However, cordycepin administration to diabetic mice could mitigate this effect (Fig. 1A). There was no significant difference in exploration time among all the groups (Fig. 1B).

The effects of cordycepin on the levels of oxidative stress markers

The GSH level was significantly lower in diabetic mice than in nondiabetic mice. However, cordycepin treatment greatly mitigated these effects (Fig. 2A). There was no significant difference in CAT or SOD levels among the groups (Fig. 2B, 2C).

Identification of differentially expressed proteins in the brain by LC–MS/MS analysis

A total of 1,455 proteins were identified. Of these, 882 were present in all four groups. The Venn diagram in Fig. 3 shows the number of differentially expressed proteins among the groups. There were 34, 13, 5, and 1 proteins uniquely expressed in the N, N+Cor, DM, and DM+Cor groups, respectively.

Functional annotations of the 13 and 1 proteins uniquely expressed in the N+Cor and DM+Cor groups, respectively, were obtained using the Panther program (Fig. 4). In the biological processes category, the proteins uniquely expressed in the N+Cor group were associated with cellular process (19.0%), biological regulation (14.3%), cellular component organization or biogenesis (14.3%), metabolic process (14.3%), multicellular organismal process (9.5%), response to stimulus (9.5%), development process (4.8%), immune system process (4.8%), multi-organism process (4.8%), and signaling (4.8%).
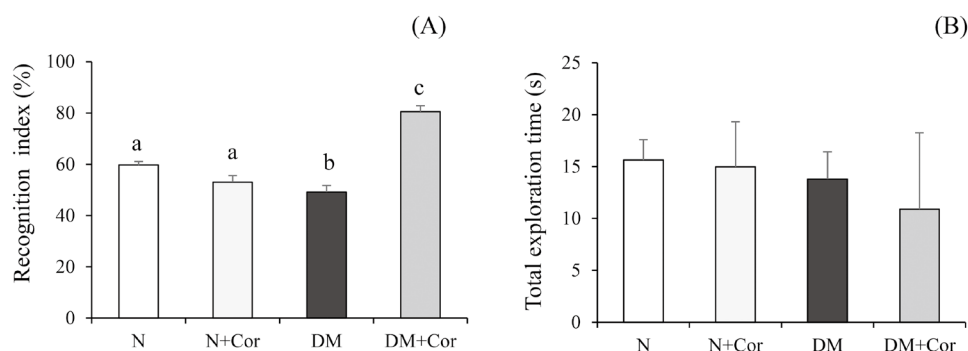

Fig. 1. The effect of cordycepin on cognition in streptozotocin (STZ)-induced diabetic mice as assessed by the novel object recognition (NOR) test. **(A)** Recognition index. **(B)** The total exploration time for both trials combined. Values with a different letter above the bar differ significantly ($P<0.05$). N, healthy control mice treated with vehicle; N+Cor, healthy mice treated with cordycepin at a dose of 24 mg/kg body weight; DM, diabetic mice treated with vehicle; DM+Cor, diabetic mice treated with cordycepin at a dose of 24 mg/kg body weight.
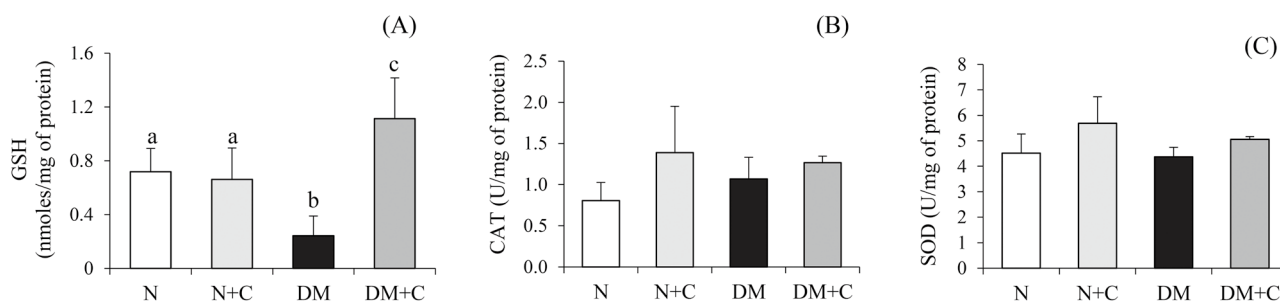
Fig. 2. Effect of cordycepin on oxidative stress markers. **(A)** Reduced glutathione (GSH) levels. **(B)** Catalase (CAT) levels. **(C)** Superoxide dismutase (SOD) levels. Values with a different letter above the bar differ significantly ($P<0.05$). N, healthy control mice treated with vehicle; N+Cor, healthy mice treated with cordycepin at a dose of 24 mg/kg body weight; DM, diabetic mice treated with vehicle; DM+Cor, diabetic mice treated with cordycepin at a dose of 24 mg/kg body weight.
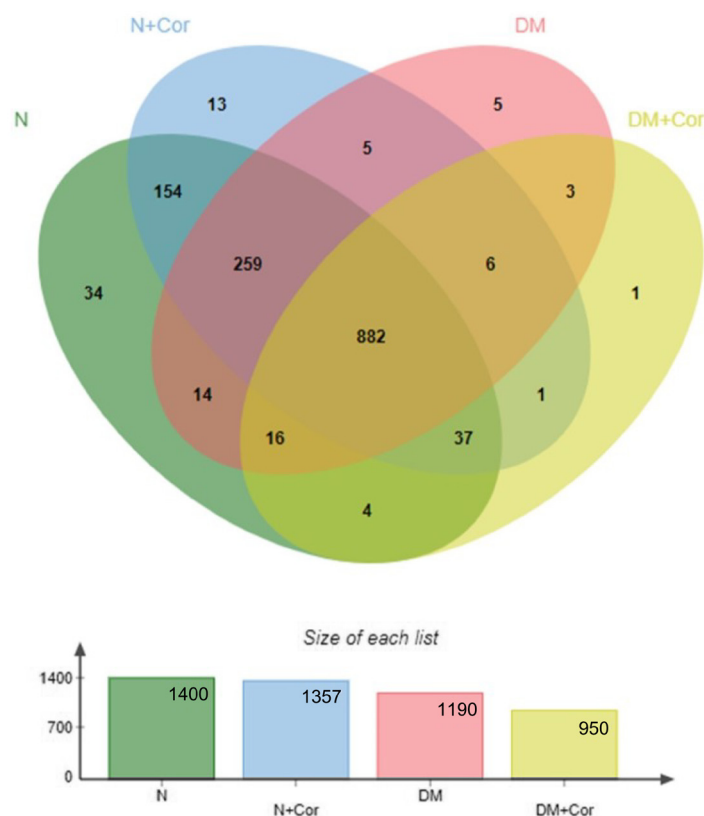
Fig. 3. Venn diagram of the protein expression profiles among the four groups. N, healthy control mice treated with vehicle; N+Cor, healthy mice treated with cordycepin at a dose of 24 mg/kg body weight; DM, diabetic mice treated with vehicle; DM+Cor, diabetic mice treated with cordycepin at a dose of 24 mg/kg body weight.
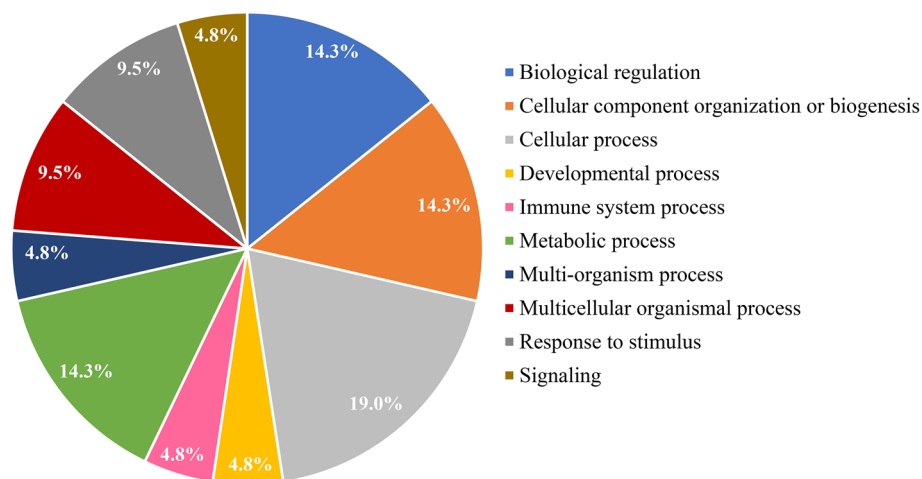
Fig. 4. Distribution of the proteins uniquely expressed in the N+Cor group (healthy mice treated with cordycepin at a dose of 24 mg/kg body weight) in the Biological Process category.

Validation of proteins uniquely expressed in the brains of mice in the DM+Cor group and those shared between the N+Cor and DM+Cor groups

The one protein found to be expressed only in the DM+Cor group was identified as serine/threonine-protein kinase ULK1, isoform 2, in UniProt. ULK1 is associated with autophagy, axon extension, axonogenesis, and neuron projection development (Table 2). One of the proteins found to be shared between the N+Cor and DM+Cor groups was glutamyl-tRNA synthetase (EARS2), which functions in glutamyl-tRNA aminoacylation (Table 3).

Protein interaction analysis of proteins uniquely expressed in the brains of mice in the DM+Cor group and those shared between the N+Cor and DM+Cor groups

The proteins and chemicals that interact with the uniquely expressed proteins ULK1 and EARS2 were analyzed by STITCH (Fig. 5). Numerous proteins in pathways such as regulation of autophagy, aminoacyl-tRNA biosynthesis, AMP-activated protein kinase (AMPK) signaling pathway, and neurotrophin signaling pathway have been reported to interact with ULK1 and EARS2.

DISCUSSION

It is increasingly clear that the brain is also a site of diabetic end-organ damage. Cognitive decline has been listed as one of the many complications of diabetes, along with retinopathy, nephropathy, and cardiovascular disease [45, 67]. Although cognitive decline in DM has a limited effect on the quality of life at the early stage, patients with DACD are at an increased risk of developing dementia, and early prevention is regarded as the most effective therapy to prevent the progression to dementia in DM patients [81]. Owing to their perceived effectiveness, fewer side effects, and relatively low costs, the use of traditional foods and medicine derived from natural sources has gained increasing interest as a means of controlling DM [60, 63].

Cordycepin (3′-deoxyadenosine) is the major bioactive component of *C. militaris*, a fungus used in traditional Chinese medicine. Cordycepin has a variety of biological properties, including antioxidant, anti-inflammatory, and neuroprotective effects [9, 11, 51, 58, 79]. Although some studies have investigated the neuroprotective actions of cordycepin in animal models, the effect of this compound on cognitive decline in STZ-induced diabetic mice remains unknown. Consequently, the aim of this study was to

Table 2. Identified unique protein in cordycepin-treated diabetic mice (DM+Cor)

Accession No.	Gene name	Protein name	Function	Peptide sequence	ID score
gi|40254402	ULK1	Serine/threonine-protein kinase ULK1	Autophagy; axon extension; axonogenesis; neuron projection	DSGGSSK	14.1

Table 3. Identified shared protein in cordycepin-treated control mice (N+Cor) and cordycepin-treated diabetic mice (DM+Cor)

Accession No.	Gene name	Protein name	Function	Peptide sequence	ID score
gi|852738019	Ears2	Glutamyl-tRNA synthetase	Glutamyl-tRNA aminoacylation	LELLKKEALRSYQTPR	20.41
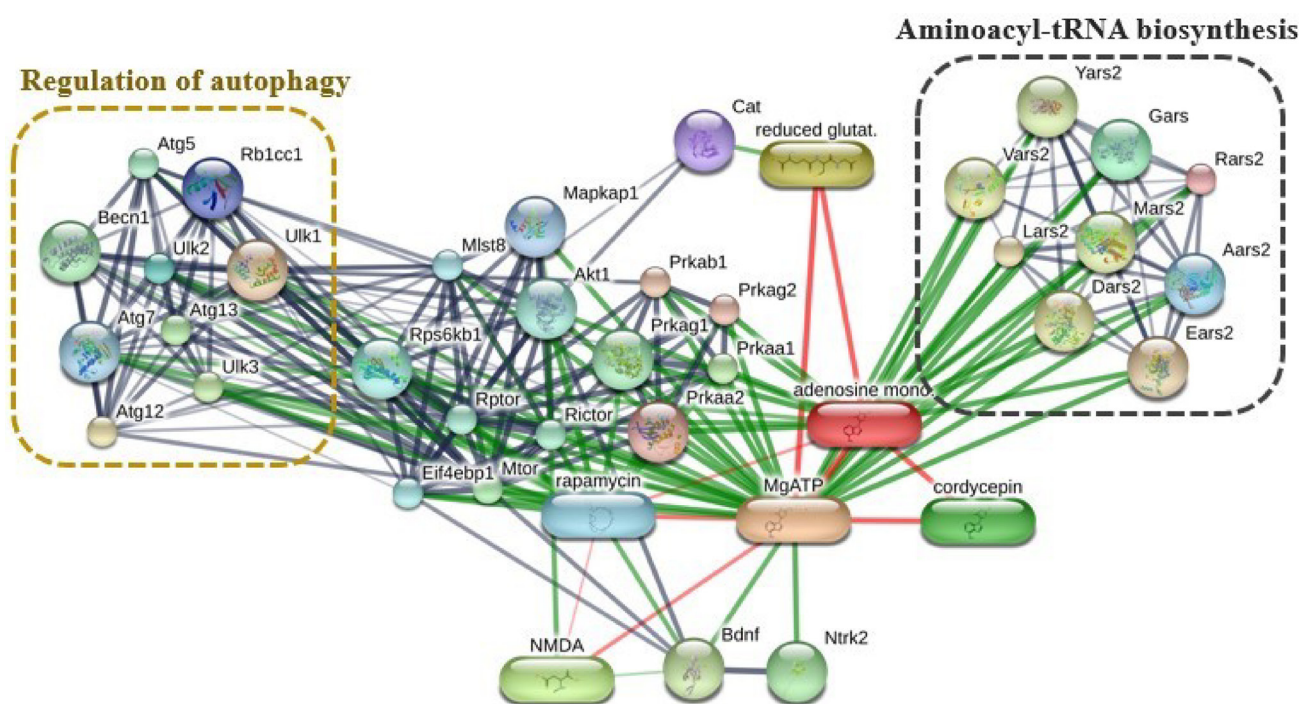
Fig. 5. The protein interaction network of cordycepin and unique brain protein (ULK1 and Ears2) in the regulation of autophagy and the aminoacyl-tRNA biosynthesis pathway generated using STITCH.

investigate the levels of oxidative stress markers and protein expression associated with cognition in the brains of cordycepin-treated diabetic mice.

Hyperphagia and polydipsia are so common in DM [57]. In support of this view, both of food intake and water intake significantly increased in diabetic mice (Table 1). However, cordycepin treatment significantly decrease in food and water intake in DM mice which agreement with previous reports [39, 42]. It has been reported that the type 1 DM has low concentration of leptin, which caused to hyperphagia [12, 46]. The Janus tyrosine kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling plays a major role in leptin's regulation of food intake [40]. Previous studies found that cordycepin treatment improved the phosphorylation of JAK2 and STAT3 protein which leads to reduce the food intake [36, 49, 72]. This is clearly that cordycepin could reduce the food intake *via* JAK2/STAT3 signaling pathway in DM mice. The results of the present study demonstrated that mice with STZ-induced DM displayed a significant decrease in body weight (Table 1). DM has been reported to affect body weight as a result of protein degradation in muscular tissue due to insulin deficiency [15]. The reduction of food and water intake might be associated with reduction of the body weight. Addition, numerous studies showed the effect of cordycepin on the suppression of body weight. Cordycepin can activate AMPK *via* interaction with the key $\gamma 1$ regulatory subunit, which accounts for more than 80% of total AMPK activity in most tissues [18, 74]. When activated, its decreased fatty acid levels by phosphorylating and inhibiting acetyl-CoA carboxylase (ACC) which is a critical enzyme for controlling fatty acid biosynthesis and oxidation [17]. In support of this view, cordycepin can reportedly inhibit adipogenesis, suppress lipid accumulation and reduced body weight *via* the AMPK activation [17, 65]. Addition, cordycepin treatment can reduced the body weight through regulating gut microbiota in obese rats [4]. This study is in agreement with previous reported that showed cordycepin treatment could reduce the body weight in both of normal and DM mice. We found that DM mice had significantly higher blood glucose levels compared with those of control mice (Table 1). This result was similar to that of Yi *et al*. [78], who reported that STZ treatment led to increased blood glucose levels due to the destruction of β-cells, resulting in insulin deficiency and, finally, hyperglycemia. Previous study found 14 days of cordycepin treatment could reduce blood glucose level in alloxan-induced diabetic mice [42]. However, we found that cordycepin supplementation could not reduce blood glucose levels in STZ-induced diabetic mice. It has been reported that the reversal of the diabetic state of alloxan is greater than STZ-induced diabetic state [20, 21]. Addition, the hypoglycemic effect of cordycepin is a time dependent manner [42, 80]. Consequently, 14 days of cordycepin treatment are not enough for reduced blood glucose levels by STZ induction. In agreement with previous findings [15, 50], our results showed that STZ-induced DM led to cognitive impairment (Fig. 1A). However, cordycepin supplementation to diabetic mice enhanced their memory ability as evidenced by the results of the NOR test and the significantly higher RI compared with that of nontreated DM mice. *C. militaris* extract can attenuate learning and memory behavior in animal models [24, 26, 53], while oral cordycepin (10 mg/kg) administration can improve learning and memory performance in ischemic mice [9].

Cordycepin reportedly exhibits marked antioxidant and radical-scavenging activities [1]. GSH is a key endogenous nonenzymatic element of the antioxidant system, playing an important role both as a substrate for glutathione peroxidase and as a direct scavenger of free radicals [13]. Our results indicated that GSH levels were significantly decreased in the brains of DM mice (Fig. 2A), which is in agreement with a previous finding [2, 41, 67]. In this study, we showed that cordycepin treatment significantly increased GSH levels in DM mice. Several studies have reported that cordycepin regulates ROS generation through NADPH oxidase [27, 79]. Additionally, the pentose in the nucleotide of cordycepin might take part in the pentose phosphate pathway to produce NADPH, which could attenuate free radical-induced damage [25]. However, we found no significant differences in CAT and SOD activity between DM and control mice (Fig. 2B and 2C). CAT and SOD activity does not change at the onset of DM as the body's defenses act to neutralize the ROS generated. SOD activity results in hydrogen peroxide generation, which also results in the coordinated, normal activity of CAT in abrogating the DM-mediated damage [5]. Here, we found that cordycepin treatment slightly tendency increased CAT and SOD activity in both the control and DM mice. Other studies have reported similar results, namely, that cordycepin can upregulate CAT and SOD levels through the AMP-activated protein kinase (AMPK)-FOXO3a signaling pathway [31].

Proteomics is increasingly used as a means of assessing protein expression [66]. In the present study, we identified EARS2 as a protein expressed in both cordycepin-treated control mice and cordycepin-treated diabetic mice (Table 3). EARS2 is a member of the aminoacyl-tRNA synthetase (aaRSs) protein family, comprising a group of nuclear-encoded enzymes that ensure the correct translation of the genetic code by conjugating each of the 20 amino acids to their cognate tRNA molecule [7]. EARS2 can covalently link the amino acid glutamate to its cognate $tRNA^{Glu}$ [29]. Glutamate is the major excitatory neurotransmitter, and is involved in neuronal growth, synaptic plasticity, and the facilitation of cognitive function and learning [14, 52]. Several studies have reported that impaired glutamate signaling is associated with cognitive dysfunction [56]. Additionally, chronic supplementation of glutamate at adequate amounts can improve memory performance, including spatial, recognition, and associative memory processes [64]. Interestingly, in the present study, ULK1, a serine/threonine-protein kinase, was the only protein expressed uniquely in diabetic mice treated with cordycepin (Table 2). ULK1 activity is required for autophagy, an intracellular catabolic system that helps in systematic degradation to maintain homeostasis [6]. There are three main forms of autophagy, namely, chaperone-mediated autophagy, microautophagy, and macroautophagy [75]. Macroautophagy (hereafter termed as autophagy) has been the best studied due to it is the most pathway involving multiple processes with several vesicular fusion events [6]. AMPK plays a critical role in regulating autophagy either by inactivating mTORC1 or by directly phosphorylating ULK1 at Ser317, Ser555, and Ser777 [28, 38, 62]. ULK1 activation can promote the phosphorylation of beclin 1 at Ser14, which is an essential step in the initiation of autophagy. Beclin 1 promotes the lipidation of microtubule-associated protein 1 light chain 3 (LC3), yielding LC3-II, its autophagosome-associated form. Finally, autophagosomes are linked by adaptor proteins such as P62

to deliver aggregated proteins to lysosomes for degradation [28]. Normally, the autophagy is at a low level under physiological conditions [68]. In disease conditions especially in DM, a large number of abnormally folded proteins, impaired mitochondria, and free radicals caused to proteins and organelle damaged and produce toxic effects on cell [6]. Therefore, activation of autophagy is an acute response of damaged cells and helps to protecting cell [75]. There have many studies found that cordycepin could ameliorated the disease, especially in DM complications, *via* inducing autophagy both of *in vitro* and *in vivo* [10, 22, 43, 68]. As previous described, cordycepin could inducing autophagy *via* AMPK phosphorylation [18, 74]. Several studies have recently indicated that activation of autophagy *via* activated AMPK and phosphorylated ULK1 can prevent/ameliorate cognition impairment in rodents [28, 62, 70]. Addition, reduced autophagy has been associated with cognitive decline *via* the overabundance of dendritic spines on hippocampal neurons [76].

This study, NOR test is a behavioral test to study the long-term memory of mice [8]. A large body of evidence supports that the long-term memory requires the synthesis of new proteins for synaptic remodeling and morphological change. Therefore, degradation of proteins by autophagy could improve the long-term memory in animal models [19]. We found that ULK1 was uniquely expressed in diabetic mice treated with cordycepin, which suggests that cordycepin enhanced the activation of autophagy, thereby ameliorating cognitive function during DACD. Enhancement of cognitive function in diabetic mice treated with cordycepin due to acute response to clear the aggregated protein and synthesis of the new proteins for synaptic remodeling [61]. However, longer period of cordycepin treatment need to be the further study.

In conclusion, the results of our study indicated that cordycepin can ameliorate DM-associated oxidative stress and cognitive decline in STZ-induced diabetic mice. However, the molecular mechanism underlying the neuroprotective role of cordycepin in DACD remains unclear and requires further study.

CONFLICT OF INTEREST. The authors declare no conflict of interest in the present study.

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