

# Protective effect of *Cordyceps militaris* against hydrogen peroxide-induced oxidative stress *in vitro*

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**BACKGROUND/OBJECTIVES:** Excessive production of reactive oxygen species (ROS) such as hydroxyl ( $\cdot\text{OH}$ ), nitric oxide (NO), and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is reported to induce oxidative stress. ROS generated by oxidative stress can potentially damage glial cells in the nervous system. *Cordyceps militaris* (CM), a kind of natural herb widely found in East Asia. In this study, we investigated the free radical scavenging activity of the CM extract and its neuroprotective effects in  $\text{H}_2\text{O}_2$ -induced C6 glial cells.

**MATERIALS/METHODS:** The ethanol extract of CM (100-1,000  $\mu\text{g}/\text{mL}$ ) was used to measure DPPH,  $\cdot\text{OH}$ , and NO radical scavenging activities. In addition, hydrogen peroxide ( $\text{H}_2\text{O}_2$ )-induced C6 glial cells were treated with CM at 0.5-2.5  $\mu\text{g}/\text{mL}$  for measurement of cell viability, ROS production, and protein expression resulting from oxidative stress.

**RESULTS:** The CM extract showed high scavenging activities against DPPH,  $\cdot\text{OH}$ , and NO radicals at concentration of 1,000  $\mu\text{g}/\text{mL}$ . Treatment of CM with  $\text{H}_2\text{O}_2$ -induced oxidative stress in C6 glial cells significantly increased cell viability, and decreased ROS production. Cyclooxygenase-2 and inducible nitric oxide synthase protein expression was down-regulated in CM-treated groups. In addition, the protein expression level of phospho-p38 mitogen-activated protein kinase (p-p38 MAPK), phospho-c-Jun N-terminal kinase (p-JNK), and phospho-extracellular regulated protein kinases (p-ERK) in  $\text{H}_2\text{O}_2$ -induced C6 glial cells was down-regulated upon CM administration.

**CONCLUSION:** CM exhibited radical scavenging activity and protective effect against  $\text{H}_2\text{O}_2$  as indicated by the increased cell viability, decreased ROS production, down-regulation of inflammation-related proteins as well as p-p38, p-JNK, and p-ERK protein levels. Therefore, we suggest that CM could play the protective role from oxidative stress in glial cells.

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## INTRODUCTION

The occurrence of oxidative stress is relevant to both free radicals and reactive oxygen species (ROS) [1,2]. Free radicals, such as superoxide ( $\cdot\text{O}_2^-$ ), hydroxyl radical ( $\cdot\text{OH}$ ), nitric oxide (NO), and the non-radical oxidizing agents like hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) can generate ROS. Actually, under homeostasis, ROS participates in a series of cellular functional reactions. However, in an excessively exposed situation, ROS becomes toxic and increases oxidative damage, especially being harmful for cells [3-5]. Cellular damage by oxidative stress disrupts the normal mechanisms of cellular signaling [6,7]. According to Gille and Joenje [8], disturbing the prooxidant-antioxidant balance is a way to induce oxidative stress. Generally,  $\text{H}_2\text{O}_2$  itself is stable in culture media; however, it can generate toxic  $\cdot\text{OH}$  in the cell through the transition metal-catalyzed reaction. Although cellular catalase has been reported to decompose  $\text{H}_2\text{O}_2$  to water and oxygen to prevent  $\cdot\text{OH}$  formation, the function of catalase

might loss balance when treated with high doses of  $\text{H}_2\text{O}_2$  and the catalase activity is inversely correlated with the cytotoxic effect of  $\text{H}_2\text{O}_2$  [9]. The exposure of cells to  $\text{H}_2\text{O}_2$  results in DNA damage and eventually causes cellular apoptosis or/and induces inflammatory responses [10]. These alterations of homeostasis cause cellular damage by  $\text{H}_2\text{O}_2$ -induced oxidative stress. In the central nervous system, glial cells play a crucial role in protecting neurons [11,12]. Oxidative stress in glial cells is closely related to neurodegenerative dysfunctions such as Alzheimer's disease (AD) and Parkinson's disease [13,14]. Therefore, research on the regulation of glial cell function and protection from oxidative stress has gained interest.

Oxidative stress occurs with inflammation and activates apoptosis pathways [12,15]. Inflammatory cells release cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) mediators, which are associated with age-related degenerative diseases [16,17]. Inflammatory cytokines activate mitogen-activated protein kinases (MAPKs) pathways, including c-Jun

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NH<sub>2</sub>-terminal kinase (JNK) and p38 MAPK, which are important for cellular proliferation and apoptosis. Moreover, the phosphorylated JNK and p38 are known to regulate apoptotic cell death [18].

The edible mushroom *Cordyceps militaris* (CM) is a kind of traditional medical herb widely used for hundred years in Asia. Several studies have shown that CM has exerted functions on immunomodulatory, neuroprotection, anti-bacterial and anti-tumor properties [19-21]. A previous research indicated the protective effect of CM against oxidative damage under high glucose induction conditions [22]. According to Ng and Wang [23], the *Cordyceps* species were reported to contain several active compounds, including cordycepin, ergosterol, and polysaccharides. Of these, the antioxidant property of cordycepin isolated from CM (3'-deoxyadenosine, C<sub>10</sub>H<sub>13</sub>N<sub>5</sub>O<sub>3</sub>) has been studied recently [24,25]. Ramesh *et al.* [24] have reported that cordycepin attenuates oxidative stress and exerts antioxidant capacity *in vivo*. Moreover, polysaccharides isolated from CM have also demonstrated to inhibit mitochondrial injury and improve immune responses by scavenging ROS [26,27]. In addition, CM has been reported to inhibit ROS generation and protect against oxidative stress induced in human dermal fibroblast cells [28]. The present study aimed to investigate the protective effect of CM against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and explore the related molecular mechanisms against oxidative stress and apoptosis in C6 glial cells.

## MATERIALS AND METHODS

### Sample and experimental groups

The ethanol extract of CM was obtained from the National Institute of Horticultural and Herbal Science (RDA, Jeonbuk-do, Korea). CM was dissolved in dimethyl sulfoxide (DMSO) to prepare a stock solution and further diluted with Dulbecco's modified Eagle's medium (DMEM) before the experiment. The experimental groups of C6 glial cells were categorized as follows:

- (1) Normal: no treatment with CM or H<sub>2</sub>O<sub>2</sub>;
- (2) Control: treatment with 300 μM H<sub>2</sub>O<sub>2</sub>;
- (3) 0.5 μg/mL: treatment with CM (0.5 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM);
- (4) 1 μg/mL: treatment with CM (1 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM);
- (5) 1.25 μg/mL: treatment with CM (1.25 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM);
- (6) 2.5 μg/mL: treatment with CM (2.5 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM).

### Reagents

Griess reagent, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2-deoxyribose, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dichlorofluorescein diacetate (DCF-DA), and DMSO were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were obtained from Acros Organics (Kangnam-gu, Seoul, Korea) and Kanto Chemical Co. (Tokyo, Japan), respectively. H<sub>2</sub>O<sub>2</sub> and sodium pentacyanonitrosylferrate (III) dihydrate (SNP) were purchased from Junsei Chemical Co. (Tokyo, Japan). DMEM, fetal bovine serum (FBS), and penicillin-streptomycin were acquired from Welgene (Daegu, Korea). Radioimmunoprecipitation assay (RIPA) buffer and the protein marker were obtained from Elpis

Biotech (Daejeon, Korea). The polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Billerica, MA, USA). Primary and secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, MA, USA), respectively.

### DPPH assay

The DPPH radical scavenging assay was performed as described previously by Hatano *et al.* [29]. Sixty micromoles of the DPPH solution were added following treatment of the sample (100, 250, 500, 1,000 μg/mL). The absorbance was measured at 540 nm following incubation for 30 min at room temperature.

$$\text{DPPH radical scavenging activity (\%)} \\ = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}} / \text{Abs}_{\text{control}}) \times 100$$

### ·OH assay

The scavenging activity of the ·OH radical was measured according to the method described by Chung *et al.* [30]. The CM extract was mixed with a solution of 10 mM FeSO<sub>4</sub>·H<sub>2</sub>O-EDTA, 10 mM 2-deoxyribose, and 10 mM H<sub>2</sub>O<sub>2</sub>. The sample mixture was incubated at 37°C for 4 h and was added to a solution containing 1.0% TBA and 2.8% TCA. The solution was boiled for 20 min and cooled in ice bath. The absorbance was measured at 490 nm.

$$\cdot\text{OH radical scavenging activity (\%)} \\ = [1 - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}}) / (\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}})] \times 100$$

### NO assay

The scavenging activity of the NO radical was measured according to the method described by Marcocci *et al.* [31]. The sample (100, 250, 500, 1,000 μg/mL) was mixed with 5 mM SNP solution and incubated for 150 min [31], after which it was mixed with Griess reagent in a ratio of 1:1 and incubated at room temperature for 30 min. The absorbance was measured at 540 nm.

### Cell culture

C6 glial cells were obtained from the Korea Cell Line Bank (Seoul, Korea) and were cultured in T75-flasks using DMEM, 10% FBS, and 100 U/mL penicillin-streptomycin at 37°C under an atmosphere of 5% CO<sub>2</sub>. For subculture, the cell culture medium was replaced daily by using 0.05% trypsin-EDTA.

### Measurement of cell viability

Cell viability was determined using the MTT assay as described by Mosmann [32]. C6 glial cells were seeded in a 96-well plate at a density of 5 × 10<sup>4</sup> per well and incubated for 24 h. Different concentrations of CM (0.5, 1, 1.25, 2.5 μg/mL) was administered to the cells for 1 h after which H<sub>2</sub>O<sub>2</sub> (300 μM) was added. Following overnight incubation, the cell culture media was replaced with MTT solution and the plate was incubated for 30 min. Finally, cell viability was determined by measuring absorbance at 540 nm.

### Measurement of ROS production

DCF-DA solution was used as a fluorochrome in ROS

generation according to the method from Ma *et al.* [33]. At a concentration of  $5 \times 10^4$  cells/mL, C6 glial cells were seeded into a 96-well plate and incubated for 24 h. The cells were treated with CM (0.5, 1, 1.25, 2.5  $\mu\text{g}/\text{mL}$ ) for 1 h, following which 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  was added. Finally, DCF-DA (80  $\mu\text{M}$ ) was added to the cells followed by incubation for 30 min. ROS production in cells was monitored by FLUO star OPTIMA (BMG labtech, Ortenberg, Germany) at excitation and emission wavelengths of 480 nm and 535 nm, respectively.

#### Western blotting

The cells were washed with phosphate-buffered saline (PBS) after aspiration of the culture medium. Thereafter, the cells were lysed in lysis buffer containing a mixture of RIPA buffer and 1 $\times$  protease inhibitor cocktail. For western blotting, an equal amount of proteins was loaded in each lane. Fifteen micrograms of total protein were separated using a 10% or 13% SDS-polyacrylamide gel and were transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk and incubated overnight at 4°C with primary antibodies [COX-2 and iNOS (1:200, Calbiochem); p-p38 (1:1,000, Cell Signaling); p-JNK (1:200, Santa Cruz); p-ERK (1:1,000, Cell Signaling); and  $\beta$ -actin (1:1,000, Cell Signaling)]. Finally, the membranes were probed with HRP-linked secondary antibodies. Protein expression was measured using the enhanced chemiluminescence detection system (Davinci Chemi, Seoul, Korea).

#### Statistical analysis

The data are represented as the mean  $\pm$  standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA), followed by the Duncan's multiple-range test. Significance was considered at  $P < 0.05$ .

## RESULTS

#### DPPH radical scavenging activity

DPPH is widely used to measure the ability of various biological substances to scavenge free radicals [34]. As shown in Table 1, the treatment with CM extract of the concentrations from 100 to 1,000  $\mu\text{g}/\text{mL}$  showed a significant scavenging effect of DPPH radical. Moreover, the capacity on DPPH radical scavenging increased concentration-dependently.

#### $\cdot\text{OH}$ radical scavenging activity

The  $\cdot\text{OH}$  radical generated by the  $\text{H}_2\text{O}_2$ /iron system is highly reactive and unstable [35]. Table 1 showed that the CM extract

**Table 1.** DPPH,  $\cdot\text{OH}$ , and NO radical scavenging activity of *Cordyceps militaris* (CM)

Treatment ( $\mu\text{g}/\text{mL}$ )	Scavenging activity (%)		
	DPPH	$\cdot\text{OH}$	NO
0	2.75 $\pm$ 5.09 <sup>d</sup>	0.95 $\pm$ 0.73 <sup>e</sup>	1.62 $\pm$ 0.41 <sup>e</sup>
100	3.88 $\pm$ 2.29 <sup>d</sup>	90.65 $\pm$ 0.18 <sup>d</sup>	5.14 $\pm$ 1.39 <sup>d</sup>
250	25.89 $\pm$ 2.76 <sup>c</sup>	95.31 $\pm$ 0.56 <sup>c</sup>	13.62 $\pm$ 2.23 <sup>c</sup>
500	43.52 $\pm$ 2.25 <sup>b</sup>	102.47 $\pm$ 0.31 <sup>b</sup>	23.68 $\pm$ 0.65 <sup>b</sup>
1,000	59.16 $\pm$ 4.09 <sup>a</sup>	104.34 $\pm$ 0.23 <sup>a</sup>	29.68 $\pm$ 2.99 <sup>a</sup>

Values are mean  $\pm$  SD.

<sup>a-e</sup> The different letters indicate significant differences in the mean ( $P < 0.05$ ) using the Duncan's multiple-range test.

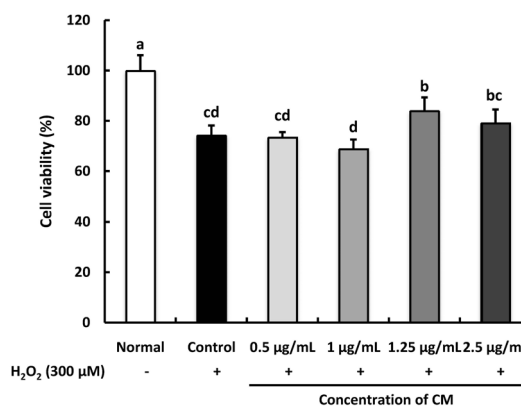
exhibited strong scavenging activity for the  $\cdot\text{OH}$  radical. The treatment of CM at the concentration of 100  $\mu\text{g}/\text{mL}$  showed 90.65%  $\cdot\text{OH}$  scavenging activity.

#### NO radical scavenging activity

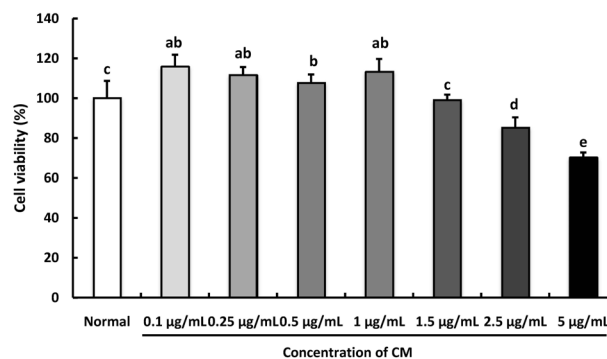
The NO radical is supposed to be related to the formation of peroxynitrite anion with high toxicity and oxidative damage [36]. Table 1 illustrated the NO radical scavenging activity of CM extract. It indicated that CM extract significantly increased NO scavenging ability in a concentration-dependent manner.

#### Effect of CM on cell viability in $\text{H}_2\text{O}_2$ -induced C6 glial cells

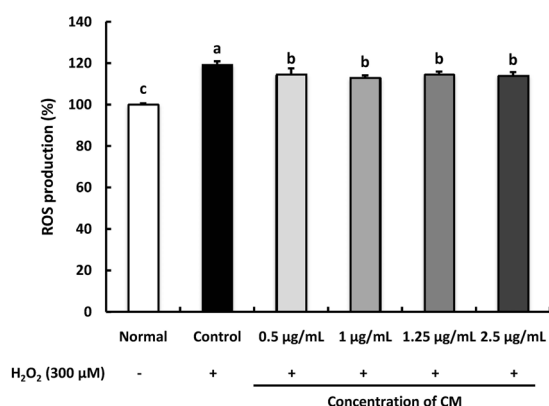
We performed the MTT cytotoxicity assay to determine the effect of CM on cell viability (Fig. 1). We assessed the toxicity of various concentrations (0.1, 0.25, 0.5, 1, 1.5, 2.5, and 5  $\mu\text{g}/\text{mL}$ ) of the CM extract in C6 glial cells. At 5  $\mu\text{g}/\text{mL}$ , the cell viability decreased to below 80%, indicating that CM might produce a toxic effect on glial cells at high doses (Fig. 2). When C6 glial cells were treated with 300  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ , the cell viability was



**Fig. 1.** Effect of *Cordyceps militaris* (CM) on  $\text{H}_2\text{O}_2$ -induced C6 glial cell viability. Cells were pre-treated with CM extract (0.5, 1, 1.25, 2.5  $\mu\text{g}/\text{mL}$ ) for 1 h, followed by the addition of 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. Values are presented as the mean  $\pm$  SD. The different letters (a-d) indicate significant differences ( $P < 0.05$ ) using the Duncan's multiple-range test. Normal: no treatment with CM or  $\text{H}_2\text{O}_2$ ; Control: treatment with 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$ ; 0.5  $\mu\text{g}/\text{mL}$ : treatment with CM (0.5  $\mu\text{g}/\text{mL}$ ) +  $\text{H}_2\text{O}_2$  (300  $\mu\text{M}$ ); 1  $\mu\text{g}/\text{mL}$ : treatment with CM (1  $\mu\text{g}/\text{mL}$ ) +  $\text{H}_2\text{O}_2$  (300  $\mu\text{M}$ ); 1.25  $\mu\text{g}/\text{mL}$ : treatment with CM (1.25  $\mu\text{g}/\text{mL}$ ) +  $\text{H}_2\text{O}_2$  (300  $\mu\text{M}$ ); 2.5  $\mu\text{g}/\text{mL}$ : treatment with CM (2.5  $\mu\text{g}/\text{mL}$ ) +  $\text{H}_2\text{O}_2$  (300  $\mu\text{M}$ ).



**Fig. 2.** Effect of *Cordyceps militaris* (CM) on  $\text{H}_2\text{O}_2$ -induced C6 glial cell viability. Cells were pre-treated with the CM extract (0.1, 0.25, 0.5, 1, 1.25, 2.5, and 5  $\mu\text{g}/\text{mL}$ ) for 1 h, followed by the addition of 300  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. Values are presented as the mean  $\pm$  SD. The different letters (a-d) indicate significant differences ( $P < 0.05$ ) using the Duncan's multiple-range test.



**Fig. 3.** Effect of *Cordyceps militaris* (CM) on the level of reactive oxygen species (ROS) in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cell. Cells were pre-treated with the CM extract (0.5, 1, 1.25, and 2.5 μg/mL) for 1 h, followed by the addition of 300 μM H<sub>2</sub>O<sub>2</sub> for 24 h. Values are presented as the mean ± SD. The different letters (a-d) indicate significant differences ( $P < 0.05$ ) using the Duncan's multiple-range test. Normal: no treatment with CM or H<sub>2</sub>O<sub>2</sub> in the C6 glial cell; Control: treatment with 300 μM H<sub>2</sub>O<sub>2</sub>; 0.5 μg/mL: treatment with CM (0.5 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM); 1 μg/mL: treatment with CM (1 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM); 1.25 μg/mL: treatment with CM (1.25 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM); 2.5 μg/mL: treatment with CM (2.5 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM).

decreased to 74.13%, whereas the normal group was observed to be 100%. However, treatment of H<sub>2</sub>O<sub>2</sub>-induced cells with CM showed a significant increase in cell viability to 83.84% and 79.09% at the concentration of 1.25 and 2.5 μg/mL, respectively.

#### Effect of CM on ROS production in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells

The ability of CM to inhibit ROS production over a time course of 1 h was determined (Fig. 3). ROS levels in the H<sub>2</sub>O<sub>2</sub>-induced

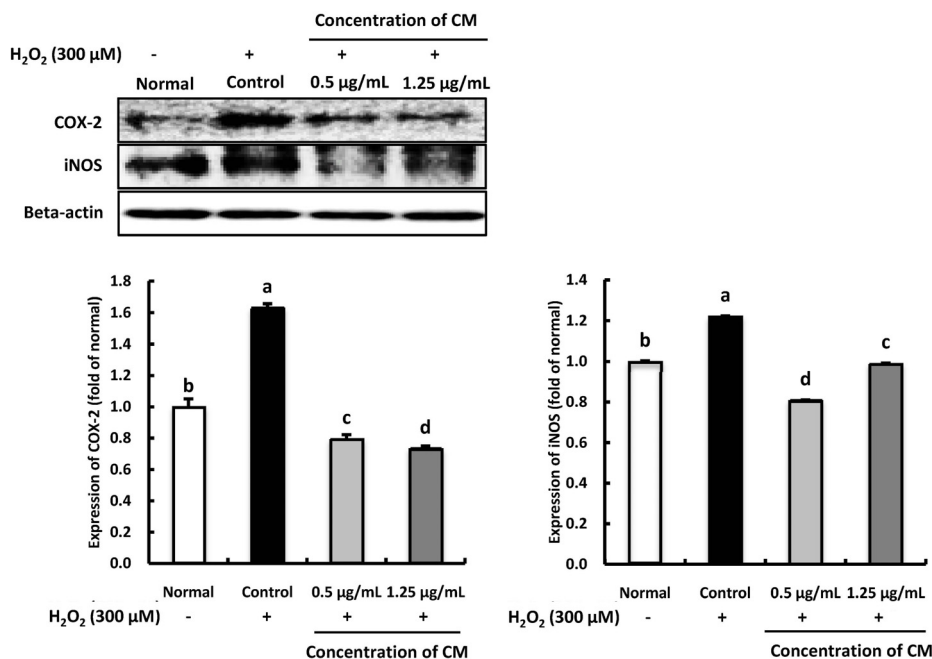
control group increased significantly than that in the normal group. Conversely, CM-treated groups significantly inhibited ROS production.

#### Effect of CM on COX-2 and iNOS protein expression in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells

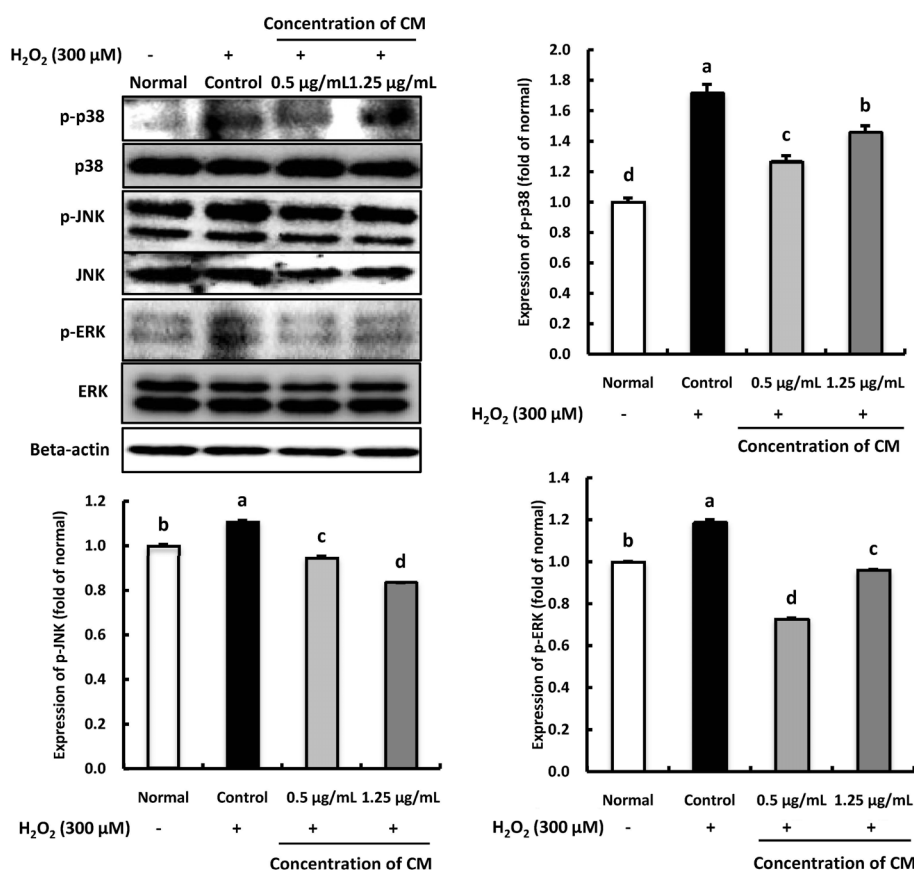
The protein expression of COX-2 and iNOS was up-regulated in the H<sub>2</sub>O<sub>2</sub>-induced control group relative to that in the normal group (Fig. 4). However, the CM-treated groups significantly inhibited COX-2 and iNOS protein expressions in the C6 glial cells. These results indicate that CM regulates the inflammatory pathway in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells by down-regulating of COX-2 and iNOS protein expressions.

#### Effect of CM on p-p38, p-JNK, and p-ERK protein expression in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells

The inhibitory effect of CM on p-p38, p-JNK, and p-ERK protein expression in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells are shown in Fig. 5. The protein levels of p-p38 and p-JNK were increased when C6 cells were treated with H<sub>2</sub>O<sub>2</sub> compared with the normal group. Moreover, compared with the normal group, p-ERK protein expression was up-regulated in the H<sub>2</sub>O<sub>2</sub>-treated control group, while the groups treated with CM significantly decreased the level of p-ERK, indicating that under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress, CM might inhibit the p-ERK expression. Furthermore, p-p38, p-JNK, and p-ERK protein expression was significantly low in the 1.25 μg/mL CM-treated group. These results suggest that CM might inhibit p-p38, p-JNK, and p-ERK protein expressions under H<sub>2</sub>O<sub>2</sub>-induced oxidative stress.



**Fig. 4.** Effect of *Cordyceps militaris* (CM) on COX-2 and iNOS protein expression in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells. Cells were pre-treated with the CM extract (0.5 and 1.25 μg/mL) for 1 h, followed by the addition of 300 μM H<sub>2</sub>O<sub>2</sub> for 24 h. Cellular protein was isolated and subjected to western blot analysis. The membrane was probed with anti-COX-2 and anti-iNOS antibodies. Beta-actin was used as an internal control. Values are presented as the mean ± SD. The different letters (a-d) indicate significant differences ( $P < 0.05$ ) using the Duncan's multiple-range test. Normal: no treatment with CM or H<sub>2</sub>O<sub>2</sub> in the C6 glial cell; Control: treatment with 300 μM H<sub>2</sub>O<sub>2</sub>; 0.5 μg/mL: treatment with CM (0.5 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM); 1.25 μg/mL: treatment with CM (1.25 μg/mL) + H<sub>2</sub>O<sub>2</sub> (300 μM).



**Fig. 5.** Effect of *Cordyceps militaris* (CM) on phospho-p38 (p-p38), phospho-JNK (p-JNK), and phospho-ERK (p-ERK) protein expression in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells. Cells were pre-treated with the CM extract (0,5 and 1,25 µg/mL) for 1 h, followed by the addition of 300 µM H<sub>2</sub>O<sub>2</sub> for 24 h. Cellular protein was isolated and subjected to western blot analysis. The membrane was probed with anti-p-p38, anti-p-JNK and anti-p-ERK antibodies. Beta-actin was used as an internal control. Values are presented as the mean ± SD. The different letters (a-d) indicate significant differences ( $P < 0,05$ ) using the Duncan's multiple-range test. Normal: no treatment with CM or H<sub>2</sub>O<sub>2</sub> in the C6 glial cell; Control: treatment with 300 µM H<sub>2</sub>O<sub>2</sub>; 0,5 µg/mL: treatment with CM (0,5 µg/mL) + H<sub>2</sub>O<sub>2</sub> (300 µM); 1,25 µg/mL: treatment with CM (1,25 µg/mL) + H<sub>2</sub>O<sub>2</sub> (300 µM).

## DISCUSSION

Free radicals are involved in neural disorders that closely related to oxidative damage [37,38]. In addition, researchers have demonstrated the correlation between oxidative stress and ROS generation, which synergistically cause degenerative diseases by inflammation and cell death [39]. Synthetic drugs are widely used as a treatment regimen for oxidative stress-related degenerative diseases, but it can cause irreversible side effects. Therefore, the use of natural medicines becomes hot topic of discussion. Dong *et al.* [40] has demonstrated antioxidant and cytotoxic properties of the methanol extract of CM against tumor cells. In addition, CM exhibits a protective effect in human dermal fibroblasts, suggesting the promising potential of CM treatment for degenerative diseases [28]. However, the neuroprotective effect of CM against oxidative stress has not been studied yet. The objective of this study was to investigate the neuroprotective effect of CM on oxidative stress-induced C6 glial cells.

Glial cells are closely related to the progression of degenerative diseases in the nervous system, including AD [41,42]. Choi *et al.* [43] has reported on the C6 glial cell as a well-established cellular model for studying ROS generation and apoptotic

pathways in neurodegenerative diseases. Impairment of neurons and glial cells by free radicals causes inflammation, which in turn promotes damage of the central nervous system [44,45]. Furthermore, inflammatory cytokines released from glial cells and cellular apoptosis are important components of neurodegenerative pathology [46]. A recent study by Yu *et al.* [47] showed that COX-2 and iNOS protein expression is up-regulated under oxidative stress by H<sub>2</sub>O<sub>2</sub> in PC12 cells - a model for neurotoxicological study - indicating that H<sub>2</sub>O<sub>2</sub> treatment induces neuroinflammation. Furthermore, rapid expression of COX-2 in glial cells may lead to neuroinflammation and affect brain function; there, COX-2 is gaining interest as a target in neurological diseases [48]. Additionally, iNOS has been shown to be involved in COX-2 regulation [49]. The present study demonstrates the role of CM in H<sub>2</sub>O<sub>2</sub>-induced inflammation in C6 glial cells by determining COX-2 and iNOS protein expression levels. The results indicate that treatment with the CM extract down-regulates COX-2 and iNOS protein expression in H<sub>2</sub>O<sub>2</sub>-induced C6 glial cells, whereas H<sub>2</sub>O<sub>2</sub> up-regulates COX-2 and iNOS expression.

To investigate the protective effect of CM against oxidative stress and inflammatory in C6 glial cells, the expressions of JNK, p-38 and ERK were observed. Treatment of glial cells with H<sub>2</sub>O<sub>2</sub>

led to neuron death by up-regulating p38 and JNK protein expression. Xie *et al.* [50] have demonstrated evidence that the signaling pathways of the phosphorylated forms of JNK and p38 contribute to neuronal dysfunction and death. Kwon *et al.* [51] have also indicated the role of JNK and p38 signaling pathways in neurodegenerative diseases. Furthermore, ERK has been shown to participate in the regulation of cell growth and differentiation, response to cellular stress, and cytokines. Choi *et al.* [52] have demonstrated the inhibitory role of cordycepin on ERK phosphorylation in LPS-stimulated RAW 264.7 cells. In this study, we observed a significant up-regulation in the protein expression levels of p-JNK, p-p38, and p-ERK in H<sub>2</sub>O<sub>2</sub>-treated glial cells; however, treatment of H<sub>2</sub>O<sub>2</sub>-induced cells with CM showed a suppressive effect on the p-JNK and p-p38 signaling pathways, suggestive of the neuroprotective ability of CM.

The present study revealed that among the various concentrations of CM (0.5, 1, 1.25, 2.5 µg/mL), 1.25 µg/mL showed protective effect against oxidative stress in C6 glial cells. In our previous study, we investigated that oral administration of the CM extract (100 and 200 mg/kg/day) attenuates amyloid beta-induced cognitive impairment in mice models. We have shown that a dose of 200 mg/kg of the CM extract significantly improves learning and memory ability without demonstrating any side effects or hepatotoxicity (data not shown). According to Shin *et al.* [53], a dose of 200 mg/kg for animals could be translated into approximately 16 mg/kg for humans (60 kg) by using *km* factor (human: 37, mouse: 3). Therefore, the daily intake dose of CM extract for humans is approximately 960 mg [53].

In conclusion, this study demonstrates that H<sub>2</sub>O<sub>2</sub>-induced cell injury is related to ROS overproduction. However, treatment with CM significantly attenuates the damage in C6 glial cells by up-regulation of COX-2 and iNOS protein expression, and down-regulation of p-JNK and p-p38 protein expression. These results show the neuroprotective effects of CM against oxidative stress. It suggested that *Cordyceps militaris* could play the protective role against oxidative stress in glial cells.

## CONFLICT OF INTEREST

The authors declare no potential conflicts of interests.

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