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Protective Effects of 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone to PC12 Cells against Cytotoxicity Induced by Hydrogen Peroxide

Ming-Yuan Su,[†] Hai-Ya Huang,[†] Lin Li,[‡] and Yan-Hua Lu*,[†]

[†]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, People's Republic of China, and [‡]Unilever Research and Development China, 33 North Fu Qian Road, Shanghai 200335, People's Republic of China

Oxidative stress has been considered as a major cause of cellular injuries in various clinical abnormalities. One of the possible ways to prevent reactive oxygen species (ROS)-mediated cellular injury is dietary or pharmaceutical therapies to augment the endogenous antioxidant defense capacity. The present study found that 2′,4′-dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC), a chalcone isolated from the buds of *Cleistocalyx operculatus*, possessed cytoprotective activity in PC12 cells treated with H_2O_2. The results showed that DMC could effectively increase cell viability [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) reduction], decrease the cell apoptotic percentage [annexin V/propidium iodide (AV/PI) assay], prevent the membrane from damage [lactate dehydrogenase (LDH) release], scavenge ROS formation, reduce caspase-3 activity, and attenuate the decrease of mitochondrial membrane potential (MMP) in PC12 cells treated with H_2O_2. Meanwhile, DMC increased the catalytic activity of superoxide dismutase (SOD) and the cellular amount of glutathione (GSH), decreased the cellular amount of malondialdehyde (MDA), and decreased the production of lipid peroxidation in PC12 cells treated with H_2O_2.

KEYWORDS: 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone; PC12 cells; oxidative stress; hydrogen peroxide; reactive oxygen species

INTRODUCTION

It is unavoidable for organisms living in an aerobic environment to be exposed to reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), the superoxide anion, and the hydroxyl radical. However, in comparison to other organs, the brain is very sensitive to oxidative stresses. On one hand, the brain has a low−moderate activity of antioxidative enzymatic defense systems. On the other hand, the brain cells consume over 20% of the oxygen used by the whole body (*1*, *2*). Oxidative damage in the brain occurs in most neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (*3*−*5*). As a major ROS, H_2O_2 causes lipid peroxidation and DNA damage in cells (*6*). H_2O_2 can be generated during amyloid aggregation, dopamine oxidation, and brain ischemia/reperfusion (*7*). Thus, formed H_2O_2 is readily converted into a highly toxic hydroxyl radical by Fenton chemistry and then further damages the lipids, proteins, and DNA. Oxidative damage could lead to mitochondrial dysfunction, calcium imbalance, and apoptosis in neuronal cells (*8*).

The rat pheochromocytoma line PC12, a clonal cell line derived from a rat adrenal medulla tumor (CRL-1721), provides a useful model system for neurological and neurochemical studies. In PC12 cells, oxidative stress induces apoptosis rather than necrosis (*8*). Increased production of ROS or a poor antioxidant defense mechanism leads to physiological dysfunction and progressive damage in the cells. Recent studies also showed that H_2O_2-induced cytotoxicity on PC12 cells, including membrane and nuclear damage, decreases mitochondrial membrane potential (MMP), superoxide dismutase (SOD) catalytic activity, and the cellular amount of glutathione (GSH), accumulates caspase-3 activation, and increases the ROS level (*9*).

To prevent or diminish ROS-induced damage, investigators have evaluated compounds extracted from plants that could prevent ROS generation and reduce neuronal damage. In recent years, studies showed that a number of products, including polyphenols, flavonoids, terpenes, and various plant extractions, exerted antioxidant actions (*10*−*12*). Some studies have demonstrated the antioxidant activities of flavonoids as hydrogen donors and free-radical scavengers and have proven their potential as chain-breaking antioxidants to inhibit the oxidation of low-density lipoprotein (*13*, *14*). Furthermore, other studies have indicated that flavonoids can inhibit free-radical formation and the propagation of free-radical reactions by chelating transition-metal ions (*15*, *16*). *Cleistocalyx operculatus* (Roxb.) Merr. et Perry (Myrtaceae) is a well-known pharmaceutical/food plant, whose buds are commonly used as an ingredient for tonic drinks in southern China. Our previous phytochemical attention to this species has led to the characterization of sterol, flavanone, chalcone, and triterpene acid from its buds (*17*). 2′,4′-Dihydroxy-6′-methoxy-3′,5′-dimethylchalcone (DMC) (**Figure 1**), the main compound from the buds of *C. operculatus*, has been proven to exhibit

*To whom correspondence should be addressed. Telephone: +86-21-64251185. Fax: +86-21-64251185. E-mail: luyanhua@ecust.edu.cn.

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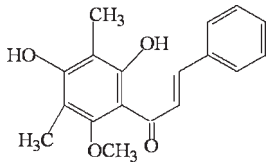

Figure 1. Chemical structure of DMC.

an anti-tumor effect both *in vitro* and *in vivo* (*18*−*20*), anti-protozoal activity (*21*), and calcium antagonist activity, which indicated that DMC may be the potential drug for diarrhea (*22*). In our previously study, the water extract of the buds of *C. operculatus* exhibited strong protective effects on lipid peroxidation in rat liver microsomes and the H_2O_2-induced trauma of PC12 cells (*23*). However, the intracellular ROS scavenging activities of DMC have not been tested. In this paper, we investigated the protective effects of DMC on PC12 cells against cytotoxicity induced by H_2O_2 *in vitro*.

MATERIALS AND METHODS

Materials. DMC was isolated from *C. operculatus* in our lab as described by Ye et al. (*17*). PC12 cell lines were purchased from the Chinese Type Culture Collection (Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from AMRESCO, Inc. (Solon, OH). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Carlsbad, CA). Calf serum was purchased from Hangzhou Sijiqing Co., Ltd. (Hangzhou, China). Annexin V (AV)-FITC/propidium iodide (PI) kit was purchased from Mbchemic (Shanghai, China). The lactate dehydrogenase (LDH) activity kit, GSH activity kit, malondialdehyde (MDA) activity kit, and SOD activity kit were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The caspase-3 activity kit was purchased from KeyGEN (Nanjing, China). 2′,7′-Dichlorfluorescein-diacetate (DCFH-DA) and Rh123 were purchased from Sigma-Aldrich Chemical (St. Louis, MO). All other chemicals were analytical reagent (AR)-grade.

Cell Culture and Treatment. PC12 cells were cultured in DMEM with 10% calf serum, 10 mmol/L HEPES, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were kept at 37 °C in a humidified atmospheric incubator with 5% CO_2/95% air. The medium was changed every other day. Before treatment, cells were plated at appropriate density on 96- or 6-well culture plates and cultured for 12 h. In all experiments, cells were preincubated with indicated concentrations of DMC or vitamin E for 30 min, and later, 150 μM H_2O_2 was added to the medium for 3 h. DMC was not removed after the addition of H_2O_2.

DMC was freshly prepared as stock solution in dimethylsulfoxide (DMSO) and diluted with DMEM before every experiment. DMSO (0.1%, v/v) had no protective or toxic effect by itself. The control group was performed in the presence of 0.1% (v/v) DMSO under the same culture conditions.

Measurement of Cell Viability. Cell survival was evaluated by the MTT assay. The MTT assay is a standard colorimetric assay, in which mitochondrial activity was measured by splitting tetrazolium salts with mitochondrial dehydrogenases in viable cells only.

PC12 cells were placed within 96-well culture plates (10^4 cells/well). After 12 h of culture, the cells were treated with DMC ranging from 0.78 to 6.25 μM for 30 min, and then, 150 μM H_2O_2 was added to the medium for 3 h. After that, the supernatant were discarded, and then, the cells were grown in 100 μL of MTT (0.5 mg/mL, dissolved in DMEM and filtered through a 0.22 μm membrane) at 37 °C. After 4 h, the formazan crystals were dissolved in 150 μL of DMSO, and the absorption values were measured at 570 nm for the reading and at 655 nm for the reference wavelength on an Automated Bio-Rad 550 microtiter plate reader (Bio-Rad Laboratories, Hercules, CA).

Morphological Study of the Cells. The PC12 cells (10^4 cells/well) were seeded in 96-well plates. They were preincubated with DMC and vitamin E for 30 min and then were treated with 150 μM H_2O_2 for 3 h. The morphologies of untreated and drug-treated cells were studied using a phase-contrast microscope (Nikon IX71 fluorescence microscope, Nikon, Inc., Tokyo, Japan).

LDH Assay. The plasma membrane damage of PC12 cells was determined by the release of LDH into the incubation medium. PC12 cells in 6-well plates were cultured and treated according to the procedures described above. After treatment, the medium were harvested for the spectrophotometrical determination of the amount of LDH released by cells using an assay kit (Nanjing Jiancheng Bioengineering Institute) according to the protocol of the manufacturer, and the absorbance of the samples was read at 490 nm.

Determination of Lipid Peroxidation. MDA, an index of lipid peroxidation, was measured by spectrophotometry using a commercial assay kit (Nanjing Jiancheng Bioengineering Institute). The assay was based on the conjugation ability of MDA with thiobarbituric acid to form a red product, which has a maximum absorbance at 532 nm. PC12 cells were incubated in 6-well plates with different concentrations of DMC or vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. After that, cells were harvested by centrifugation, washed twice with phosphate buffer solution (PBS) (pH 7.0), and then resuspended in physiological saline solution. The cells were lysed by sonication, and then the suspension was used to measure the MDA level according to the instructions of the manufacturer.

Assays for the Antioxidant System. To determine the activity of GSH and SOD, PC12 cells were incubated in 6-well plates with different concentrations of DMC or vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. The cells were harvested by centrifugation, washed twice with PBS (pH 7.0), and then resuspended in physiological saline solution. The cells were lysed by sonication, and then the suspension was used for assays according to the instructions of the manufacturer. GSH and SOD contents in cell homogenates were determined using commercial colorimetric GSH and SOD assay kits (Nanjing Jiancheng Bioengineering Institute).

Measurement of MMP. The MMP was evaluated using the fluorescent rhodamine dye, Rh123. It is a cell-permeable cationic dye that preferentially partitions into mitochondria based on the highly negative MMP (*24*). At the end of the experiments, the medium was removed and about 1×10^6 cells were harvested by trypsinization. After the cells washed twice with PBS, they were incubated with Rh123 (10 μM, Sigma) for 30 min at 37 °C in the dark. The cells were harvested and suspended in PBS. Then, the MMP was analyzed by a FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). The laser was adjusted to emit at 480 nm, and a 530 nm long-pass filter was used.

Measurement of Intracellular ROS Formation. The production of intracellular ROS was quantified using a DCFH-DA assay (*25*). Cell-permeant non-fluorescent DCFH-DA has been shown to oxidize to high-fluorescent 2′,7′-dichlorofluorescein (DCF), which is retained within the cells, in the presence of ROS. The DCF fluorescence intensity is believed to be parallel to the amount of ROS formed intracellularly. The cells on 6-well plates were incubated with 10 μM DCFH-DA (Sigma) in the DMEM in 5% CO_2/95% air at 37 °C for 30 min. After the addition of DMC (1.56−6.25 μM) or 10 μM vitamin E, the cells were incubated at 37 °C for 30 min, followed by the addition of 150 μM H_2O_2. After 3 h of incubation, the cells were harvested and suspended in PBS. The fluorescence intensity was measured by a FACScan flow cytometer at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Flow Cytometric Detection of Apoptotic Cells. The extent of apoptosis was measured through the AV-FITC/PI apoptosis detection kit (Mbchemic, Shanghai, China). Briefly, PC12 cells were incubated in 6-well plates with different concentrations of DMC or vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. The cells were harvested by centrifugation and washed twice with PBS (pH 7.0), and then, the cells were used for the AV/PI assay according to the instructions of the manufacturer. After that, it was analyzed by a FACScan flow cytometer (*26*). The fraction of the cell population in different quadrants was analyzed using quadrant statistics. Cells in the lower right (LR) quadrant represented early apoptotic cells, and cells in the upper right (UR) quadrant represented late apoptotic cells (*27*).

Measurement of Caspase-3 Activation. Caspase-3 activity was determined using a caspase-3 activity detection assay kit (KeyGEN, Nanjing, China). Briefly, after cell lysis and centrifugation at 10000*g* for

10 min at 4 °C, the supernatant from lysed cells was added to the reaction mixture containing dithiothreitol and caspase-3 substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) and further incubated for 1 h at 37 °C. Absorbance was measured on an automated Bio-Rad 550 microplate reader at 405 nm.

Statistical Analysis. All of the assays were carried out in at least three experiments with 4-fold sample. The results were expressed as the mean ± standard deviation (SD), and the significance of the differences between three and more than three groups was analyzed by one-way analysis of variance (ANOVA) followed by Dunnetts's multiple comparison test, with the scientific statistic software Origin, version 8.0.

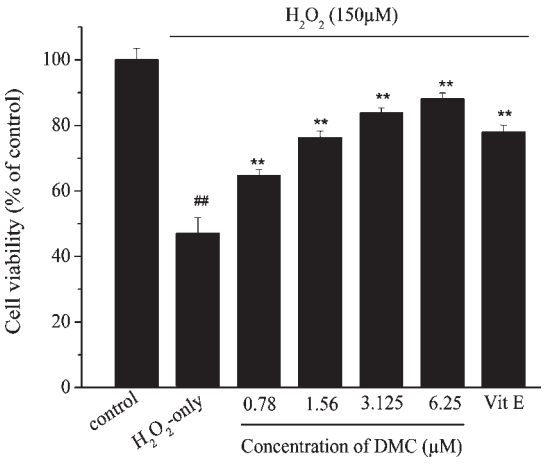

Figure 2. Cell protective effect of DMC on H_2O_2-induced cytotoxicity on PC12 cells. Cells were pretreated with 0.78−6.25 μM DMC or 10 μM vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. The viability of control cells was defined as 100%. Data shown are mean ± SD (n = 4). (##) $p < 0.01$ between control cells and only H_2O_2-treated cells. (**) $p < 0.01$ between only H_2O_2-treated cells and DMC pretreatment cells.

RESULTS

DMC Protected PC12 Cells against H_2O_2-Induced Cytotoxicity. To determine whether DMC can prevent cytotoxicity induced by H_2O_2 on PC12 cells, the connection between H_2O_2 and DMC was demonstrated. The cell viability of PC12 cells, measured by the MTT reduction assay, was decreased to 47.1% of the control group after cells were exposed to 150 μM H_2O_2 for 3 h. However, pretreatment with DMC (0.78, 1.56, 3.125, and 6.25 μM) or vitamin E (10 μM) for 30 min significantly ($p < 0.01$) elevated the cell viability of PC12 cells treated with H_2O_2 to 68.7−88.1 and 78.0%, respectively (**Figure 2**). Moreover, DMC at these concentrations alone did not cause any apparent effects on PC12 cells (data not shown).

In another study, in comparison to normal PC12 cells (**Figure 3A**), PC12 cells treated with 150 μM H_2O_2 for 3 h exhibited morphological alterations, such as cell shrinkage and membrane blebbing, which are normally associated with the occurrence of cell apoptosis (**Figure 3B**), while 6.25 μM DMC (**Figure 3C**) and 10 μM vitamin E (**Figure 3D**) pretreatment mitigated such morphological features of PC12 cells treated with H_2O_2. The results clearly indicated that the H_2O_2-induced cytotoxic effects on PC12 cells were attenuated in the presence of DMC.

Effect of DMC on the Extracellular LDH Levels in PC12 Cells. To further investigate the cytoprotective effects of DMC against H_2O_2-induced damage and cell death in PC12 cells, the LDH assay was considered as an indicator. LDH is a stable cytoplasmic enzyme in all cells, and it is rapidly released into the cell culture if the plasma membrane was damaged by oxidants. An increase of the LDH activity in the culture supernatant indicated an increase in the number of dead or plasma-membrane-damaged cells. It was showed that treatment with 150 μM H_2O_2 for 3 h caused LDH activity to increase markedly ($p < 0.01$) to 550.5% of the control group, while preincubation with DMC (0.78, 1.56, 3.125, and 6.25 μM) and vitamin E (10 μM) for 30 min significantly ($p < 0.01$) decreased the LDH release to 416.3, 361.7, 309.1, 245.6, and

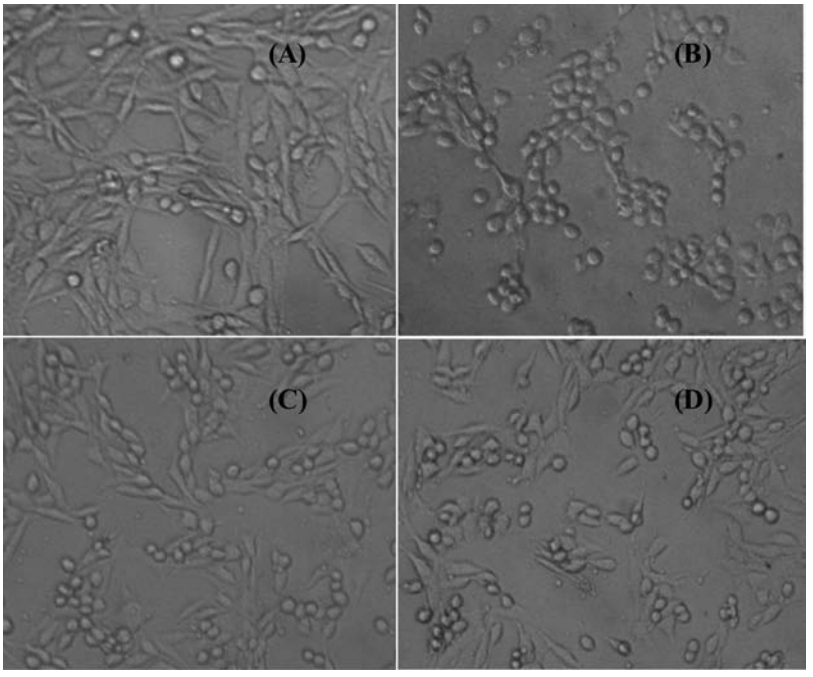

Figure 3. Effect of DMC on the morphological injury of PC12 cells induced by H_2O_2. (A) PC12 control cells. (B) PC12 cells exposed to 150 μM H_2O_2 for 3 h. There is a significant decrease in the cell number, and most of the cells demonstrated a round shape. (C and D) PC12 cells were preincubated with (C) DMC (6.25 μM) or (D) vitamin E (10 μM) for 30 min and then exposed to 150 μM H_2O_2 for 3 h. All photos were taken 3 h after exposure to H_2O_2.

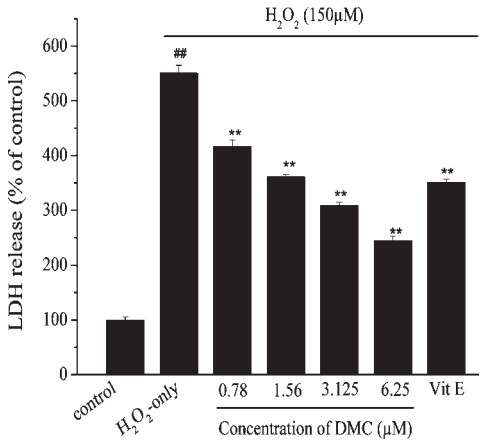

Figure 4. Inhibition of LDH release of DMC on H_2O_2-induced membrane damage in PC12 cells. Cells were pretreated with 0.78−6.25 μM DMC or 10 μM vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. LDH activity was measured using a colorimetric LDH assay kit. All data are presented as mean ± SD (n = 4). (##) $p < 0.01$ between control cells and only H_2O_2-treated cells. (**) $p < 0.01$ between only H_2O_2-treated cells and DMC pretreatment cells.

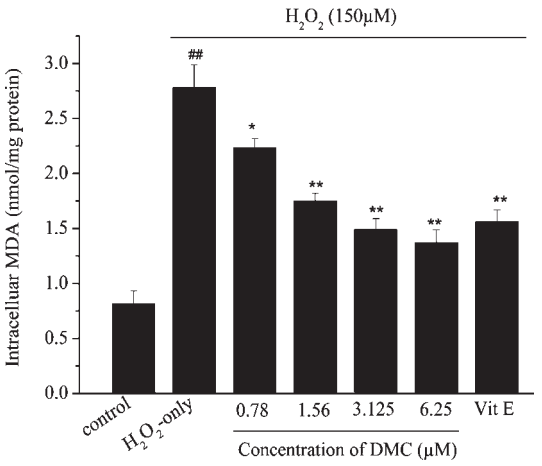

Figure 5. Effect of DMC on the MDA level in PC12 cells treated with H_2O_2. Cells were pretreated with 0.78−6.25 μM DMC or 10 μM vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. MDA activity was measured using a colorimetric MDA assay kit. All data are presented as the mean ± SD (n = 4). (##) $p < 0.01$ between control cells and only H_2O_2-treated cells. (*) $p < 0.05$ and (**) $p < 0.01$ between only H_2O_2-treated cells and DMC pretreatment cells.

351.4%, respectively, as compared to PC12 cells treated with H_2O_2 (**Figure 4**).

Effect of DMC on the Lipid Peroxidation in PC12 Cells. Lipid peroxidation is one of the earliest recognized and most extensively studied manifestations of oxygen toxicity in biology. In this study, the MDA level was quantified by the thiobarbituric acid reactive substances (TBARS) assay. A significant increase ($p < 0.01$) by 241.7% of the MDA level compared to the control group was observed in PC12 cells exposed to 150 μM H_2O_2 for 3 h (**Figure 5**). However, when the cells were pretreated with 0.78−6.25 μM DMC or 10 μM vitamin E, MDA levels were reduced ($p < 0.05$) by 19.7, 37.1, 46.4, 50.7, and 43.9% respectively, as compared to the PC12 cells treated with H_2O_2.

Effect of DMC on Antioxidant Systemic Substances in PC12 Cells. Antioxidative nutrients, such as GSH and antioxidant enzymes, such as SOD, are the primary defense substances to

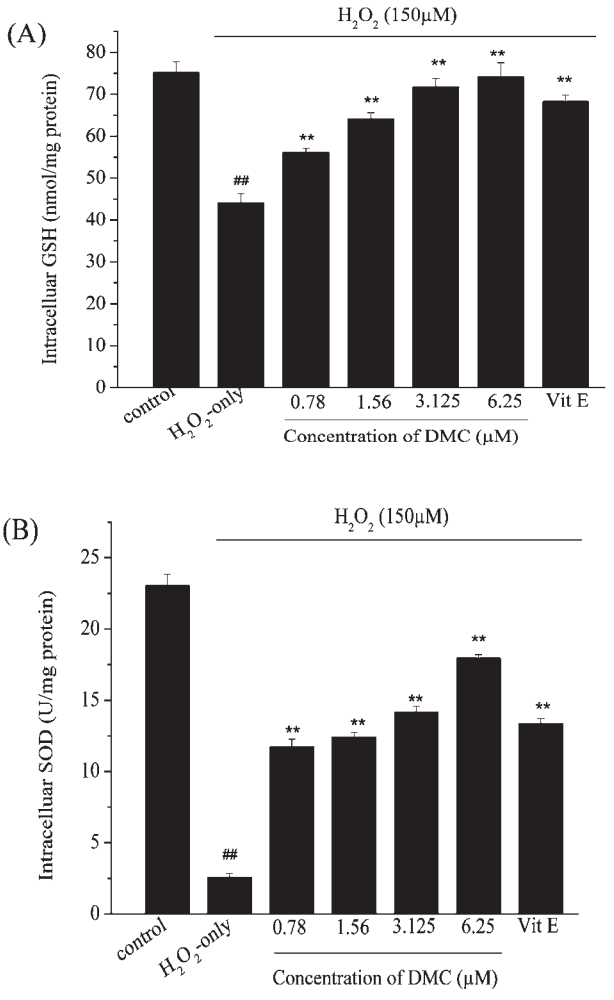

Figure 6. DMC attenuated a H_2O_2-induced decrease of antioxidant activities in PC12 cells. Cells were pretreated with 0.78−6.25 μM DMC or 10 μM vitamin E for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. The cellular amount of GSH and the catalytic activity of SOD were detected by commercial colorimetric assay kits. (A) GSH amount. (B) SOD activity. All data represent the mean ± SD (n = 4). (##) $p < 0.01$ between control cells and only H_2O_2-treated cells. (**) $p < 0.01$ between only H_2O_2-treated cells and DMC pretreatment cells.

protect living systems from oxidative damage. Exposure of PC12 cells to H_2O_2 (150 μM) for 3 h significantly ($p < 0.01$) reduced the amount of GSH (**Figure 6A**) and the activity of SOD (**Figure 6B**) to 58.7 and 11.2%, respectively, as compared to the control group. Pretreatment of PC12 cells with DMC and vitamin E for 30 min significantly ($p < 0.01$) attenuated the decrease of the GSH amount and SOD activity induced by H_2O_2.

DMC Attenuated a H_2O_2-Induced Decrease of MMP in PC12 Cells. To assess the effect of DMC on the changes of MMP induced by H_2O_2 in PC12 cells, flow cytometric analyses were carried out using Rh123. This dye is a lipophilic cation, and its uptake by mitochondria is proportional to the MMP. When PC12 cells were exposed to 150 μM H_2O_2 for 3 h, the MMP level was rapidly ($p < 0.01$) reduced to 4.6% of the control (**Table 1**). Pretreatment with DMC (1.56, 3.125, and 6.25 μM) or vitamin E (10 μM) significantly ($p < 0.01$) reduced the changes in MMP induced by H_2O_2. DMC increased the levels of MMP by 28.9−87.9%, while vitamin E increased the level of MMP by 77.3%, as compared to the PC12 cells treated with H_2O_2, which indicated that the H_2O_2-induced dissipation of the MMP in PC12 cells was partly blocked by the pretreatment with DMC.

Table 1. Effects of DMC on the Changes of the MMP Level, ROS Level, and Caspase-3 Activity in PC12 Cells Treated with H_2O_2[a]

treatment	MMP (percentage of the control)	ROS (percentage of the control)	caspase-3 activity (percentage of the control)
control	100.0 ± 2.5[b]	100.0 ± 3.1	100.0 ± 4.2
H_2O_2 (150 μM)	4.6 ± 0.3[c]	323.9 ± 5.1[c]	291.2 ± 5.9[c]
vitamin E (10 μM) + H_2O_2	81.9 ± 1.7[d]	172.0 ± 2.9[d]	146.4 ± 3.7[d]
DMC (1.56 μM) + H_2O_2	33.5 ± 2.1[d]	265.4 ± 4.8[d]	232.1 ± 3.3[d]
DMC (3.125 μM) + H_2O_2	64.7 ± 2.4[d]	224.6 ± 3.7[d]	177.6 ± 4.8[d]
DMC (6.25 μM) + H_2O_2	92.5 ± 1.9[d]	149.1 ± 4.6[d]	143.4 ± 5.3[d]

[a] Cells were pretreated with DMC (1.56−6.25 μM) or vitamin E (10 μM) for 30 min and then incubated in the presence of 150 μM H_2O_2 for 3 h. MMP, ROS, and caspase-3 were quantified as described in the Materials and Methods. [b] All data represent the mean ± SD (n = 4). [c] $p < 0.01$ between control cells and only H_2O_2-treated cells. [d] $p < 0.01$ between only H_2O_2-treated cells and DMC pretreatment cells.

DMC Inhibited a H_2O_2-Induced Intracellular ROS Increase in PC12 Cells. To determine whether DMC could prevent H_2O_2-induced ROS generation and then result in oxidative stress, levels of ROS production in the cells were determined using the flow cytometric DCFH-DA assay for measuring DCF fluorescence. Exposure of the cells to 150 μM H_2O_2 for 3 h significantly ($p < 0.01$) increased the intracellular ROS level to 323.9% of the control (**Table 1**). Such intracellular ROS accumulation was significantly ($p < 0.01$) eliminated by pretreatment with DMC or vitamin E. DMC (1.56, 3.125, and 6.25 μM) reduced the level of ROS by 58.5−174.8%, while vitamin E (10 μM) reduced the level of ROS by 151.9%, as compared to the PC12 cells treated with H_2O_2.

DMC Protected PC12 Cells against H_2O_2-Induced Apoptosis. To test whether H_2O_2-induced cell death via apoptosis, the percentage of apoptotic cells was measured by the AV/PI assay and the caspase-3 activity assay. Positive staining with AV-FITC correlates with the loss of membrane polarity, and the complete loss of membrane integrity will lead to apoptosis or necrosis. In contrast, PI can only enter cells after the loss of membrane integrity. Thus, dual staining with AV and PI allows for clear discrimination between unaffected and apoptotic cells. Our results showed that treatment of cells with 150 μM H_2O_2 for 3 h significantly ($p < 0.01$) induced apoptotic cell accumulation to approximately 46.2% (**Figure 7B**). Pretreatment of DMC (1.56, 3.125, and 6.25 μM) or vitamin E (10 μM) can significantly ($p < 0.01$) attenuate cell apoptosis to 30.3, 24.1, 17.5, and 16.7% induced by H_2O_2, respectively (panels **C**−**F** of **Figure 7**).

The apoptotic process included the activation of cysteine proteases, which represent both initiators and executors of cell death. Caspase-3 is the key apoptotic executive protein, which could be activated by both death-receptor and mitochondrial pathways. After cells were exposed to H_2O_2, the caspase-3 activity was significantly ($p < 0.01$) elevated by 191.2% of the control. When cells were pretreated with DMC, caspase-3 activity was reduced significantly ($p < 0.01$) in a dose-dependent manner, as compared to the PC12 cells treated with H_2O_2 (**Table 1**).

DISCUSSION

Eukaryotes have developed specific defenses against these ROS, including SOD, glutathione peroxidase, catalase, and a host of other proteins and peptides, whose functions are to reduce the accumulative load of ROS within the cell or intracellular space (*28*). Although antioxidant systems can help to protect the body against free radicals, they might become overwhelmed during periods of chronic oxidative stresses (*29*). Therefore, in recent years, flavonoids have generated considerable interest as potential therapeutic agents against a wide variety of chronic diseases (*30*). In the present study, we have demonstrated that DMC, the main compound from the buds of *C. operculatus*, exhibited cytoprotective effects against H_2O_2-induced damage and cell death in PC12 cells.

The oxidative-stress-induced tissue damage has been implicated in a number of disease processes, including cancer, diabetes, and neurodegenerative disorders (*2*,*31*,*32*). Free radicals, such as H_2O_2, the superoxide anion, and the hydroxyl radical, are able to produce many detrimental effects, including lipid peroxidation of cellular membranes, alteration of the lipid−protein interaction, enzyme inactivation, and DNA breakage and, in the end, to cause cell injury, necrosis, or apoptosis. As its high cellular membrane permeability, H_2O_2 is toxic and often used as a toxicant to mimic *in vitro* models of oxidative-stress-induced injury. High levels of H_2O_2 have been shown to be toxic to neurons (*25*). H_2O_2 (150 μM) in a treatment period of 3 h in our experiment resulted in substantial cell loss (**Figures 2** and **3B**).

The generation of the superoxide anion under oxidative stress reflects oxidative damage of the plasma membrane and results in lipid peroxidation. However, there are some antioxidants in cells, such as GSH and SOD, for scavenging ROS to prevent cells from damage. GSH is a small molecule scavenger, which can scavenge O^{2-}, H_2O_2, and LOOH. Meanwhile, GSH is the substrate of GSH-Px and GST, and it is necessary for these two enzymes to decomposit H_2O_2. SOD is able to transform the intracellular superoxide anion to H_2O_2, and the formed H_2O_2 is subsequently scavenged by catalase and GSH-Px through enzymatic reactions. In this study, H_2O_2 significantly ($p < 0.01$) inhibited the catalytic activity of SOD and the amount of GSH and increased the ROS level and the level of production of the lipid peroxidation, MDA in PC12 cells, whereas DMC pretreatment reversed the changes (**Figures 5** and **6** and **Table 1**), suggesting that the cytoprotective effect of DMC against H_2O_2-induced damage and cell death in PC12 cells is related to its antioxidant ability.

The protective effect of DMC was also supported by higher MMP. Mitochondria serve as final arbiters of life and death of the cell, because these organelles are not only required to generate ATP but can also trigger apoptosis or necrosis (*33*). Oxidative damage causes depletion of the materials that are necessary to produce high energy and strongly affects the electron transport chain in mitochondrial. MMP reflects the performance of the electron transport chain and can indicate a pathological disorder of this system. A higher MMP average is related to higher viability and vice versa. Mitochondrial membrane depolarization can cause cytochrome *c* to be released from the inner membrane, leading to activation of the caspase-3 cascade and apoptosis (*34*,*35*). We found that H_2O_2 led to mitochondrial membrane depolarization, while DMC pretreatment significantly ($p < 0.01$) prevented the loss of MMP (**Table 1**). The antioxidant property of DMC can be attributed to the preservation of MMP.

Caspases are cysteine proteases that mediate cell death, and caspase-3 has been shown to be an important regulator of neuronal apoptosis. Caspase-3 is cleaved and activated during the final step of apoptosis and also mediates H_2O_2-induced apoptosis by cleaving full-length poly(ADP-ribose) polymerase (PARP) (116 kDa) into an 89 kDa fragment (*36*). Our data

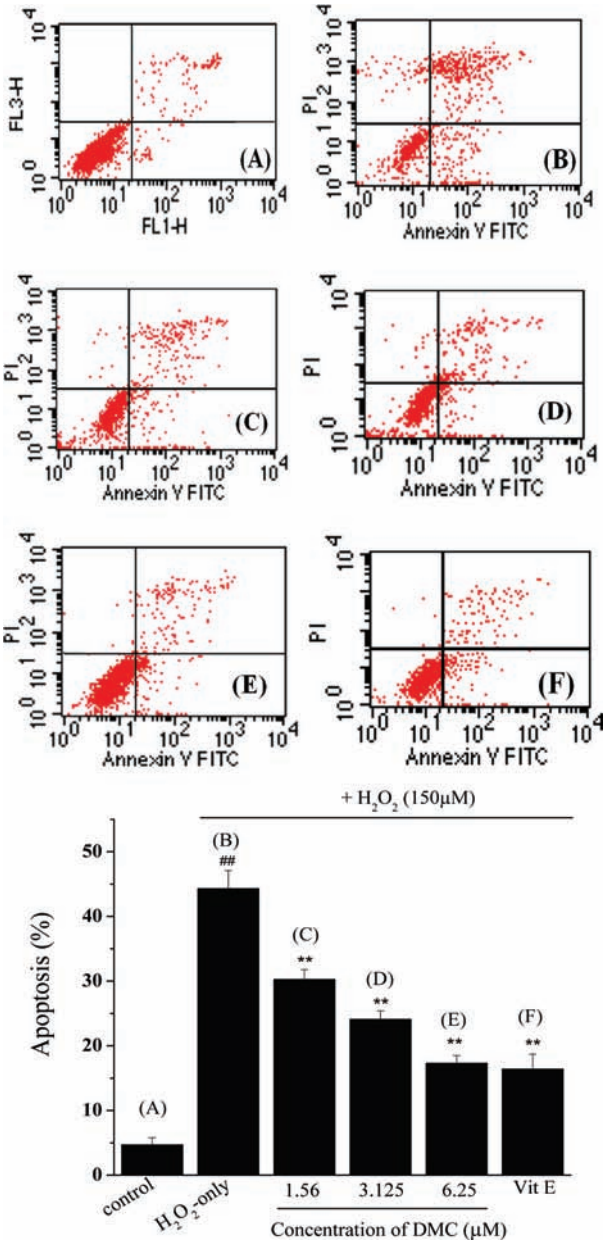

Figure 7. Inhibitory effect of DMC on H_2O_2-induced apoptosis in PC12 cells. AV-FITC/PI analysis by FCM showed the percentage of apoptotic cells in H_2O_2-induced PC12 cells with or without DMC: the sum of the UR and LR quadrants (the UR quadrant represents apoptotic cells in the early stage, and the LR quadrant represents apoptotic cells in the late stage). Cells were pretreated with DMC (1.56, 3.125, and 6.25 μM) or vitamin E (10 μM) for 30 min then incubated in the presence of 150 μM H_2O_2 for 3 h. (A) PC12 control cells. (B) PC12 cells exposed to 150 μM H_2O_2 for 3 h. (C−F) PC12 cells were preincubated with DMC (1.56, 3.125, and 6.25 μM) or vitamin E (10 μM) for 30 min, respectively, and then exposed to 150 μM H_2O_2 for 3 h. All data represent the mean ± SD ($n = 4$). (##) $p < 0.01$ between control cells and only H_2O_2-treated cells. (**) $p < 0.01$ between only H_2O_2-treated cells and DMC pretreatment cells.

showed that DMC significantly ($p < 0.01$) attenuated the caspase-3 activity in PC12 cells treated with H_2O_2 (**Table 1**), suggesting that the cytoprotective effect of DMC against H_2O_2-induced damage and cell death in PC12 cells is at least attributed to not only its antioxidant property but also the attenuation of caspase-3 activity.

In conclusion, pretreatment with DMC effectively alleviated oxidative stress and apoptosis in PC12 cells caused by H_2O_2-induced injury, suggesting that DMC may be useful in more complex intervention for chronic disease. DMC exhibited antioxidative and anti-apoptotic activities through restoring GSH and SOD activity, elevating MMP, and diminishing the MDA level, caspase-3 activity, ROS content, and LDH release. Therefore, the present results support that DMC is a potent cytoprotective agent against H_2O_2-induced damage and cell death in PC12 cells.

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