INTRODUCTION

It is well known that the production and elimination of reactive oxygen species (ROS) are in a delicate equilibrium in normal cell. Oxidative stress appears when ROS is generated beyond the scavenging capacity of the antioxidant defense system [Zhang et al., 2018d; Zhang et al., 2016]. Oxidative stress caused by excessive ROS production may disrupt the redox homeostasis, induce autophagy, trigger apoptosis, and cause irreversible tissue injury [Shen et al., 2017]. It has been confirmed that the damages caused by oxidative stress can lead to many chronic diseases such as cancer, diabetes, cardiovascular diseases, and neurodegenerative diseases [Wang et al., 2015; Zhang et al., 2016]. Therefore, how to protect the cell against oxidative stress-induced injury and enhance the cellular and tissue defenses against ROS require more attention.

Dietary intake of natural antioxidants has been demonstrated as a feasible way to eliminate the harmful effects of ROS and restore the body's antioxidant load [Seifried et al., 2007]. Hence, there is an increasing interest on natural antioxidants to prevent chronic diseases caused by oxidative stress. Food-derived protein hydrolysate, produced by the enzymatic hydrolysis of natural food proteins, is one of the numerous natural antioxidants. Due to a high antioxidant activity, satisfactory safety, and bioavailability, food-derived protein hydrolysates have received significant scientific attention in the food industry and healthcare field [Jin et al., 2013; Morifuji et al., 2010]. Protein hydrolysates from soybean [Zhang et al., 2018d], common carp [Zamora-Sillero et al., 2018], wheat germ [Zhou et al., 2016], and rice dreg [Zhang et al., 2016], exhibited potential in vitro antioxidant activity in chemical models or cell models. Additionally, our previous studies demonstrated that the protein hydrolysate (MPH) from Morchella esculenta (L.), an edible and medicinal fungus of high economic value, exhibited various antioxidant properties such as excellent reducing power, efficient free radical scavenging activity, and considerable H₂O₂ scavenging activity. Moreover, the glycosylated derivative of MPH (G-MPH) produced by conjugating with xylose via Maillard reaction exhibited higher antioxidant properties than MPH [Zhang et al., 2018b]. However, to the best of our knowledge, the cytoprotective effect of MPH and G-MPH against oxidative stress and its underlying mechanisms have never been reported.

It has been widely acknowledged that the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway is a key mediator in oxidative stress [Li et al., 2017]. Under normal conditions, Nrf2 binds to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm to form a complex. To counteract oxidative stress, Nrf2 is released from Keap1 before being translocated into the nucleus, binds to the antioxidant response elements (AREs), and promotes the expression of downstream genes including haeme oxygenase-1 (HO-1),

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NAD(P)H:quinone oxidoreductase 1 (NQO1), and some antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) [Pyo et al., 2016; Xia et al., 2017]. These enzymes play a role in cell detoxification and maintenance of antioxidant capacity which in turn help regulate the redox balance [Xia et al., 2017]. It has been reported that Maillard reaction products of fish protein hydrolysate and ribose activate Nrf2 to protect HepG2 cells against oxidative stress [Yang et al., 2017]. Therefore, it is hypothesized that MPH and G-MPH may attenuate the H$_2$O$_2$-induced oxidative injury, potentially via the activation of Nrf2 signaling pathway.

Apoptosis (programmed cell death) plays an important role in regulating numerous physiological processes such as growth, development, and homeostasis maintenance [Mańdziuk et al., 2003]. Imbalance between cell proliferation and apoptosis can cause pathological phenomena such as cancer and Alzheimer’s disease [Yoon et al., 2017; Zhou et al., 2017]. Many reports have demonstrated that various natural antioxidants protect cells from oxidative damage by suppressing apoptosis [Chen et al., 2017; Zhou et al., 2016]. However, whether the underlying mechanisms by which MPH and G-MPH exert cytoprotective effects against oxidative damage is via apoptosis inhibition or not remains unclear.

The aim of this study was thus to evaluate the cytoprotective effects of MPH and G-MPH against oxidative stress using Caco-2 cells, which is an ideal tool to assess the cellular antioxidant response to food-derived antioxidants [Ruiz-Roca et al., 2011]. Moreover, we investigated whether MPH and G-MPH exert cytoprotective effects by activating Nrf2 signaling pathway and by inhibiting apoptosis in an attempt to explain its underlying molecular mechanisms.

**MATERIALS AND METHODS**

**Reagents**

The cell culture reagents were obtained from Gibco BRL Life Technologies (USA). Malondialdehyde (MDA), glutathione (GSH), total antioxidant capacity (T-AOC), superoxide dismutase (SOD), and catalase (CAT) kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Kits for ROS assay, Annexin V-FITC detection, TUNEL assay, JC-1 assay, total protein extraction and quantification, and enhanced chemiluminescence (ECL) detection were purchased from Keygen Biotech Co., Ltd. (Nanjing, China). All primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents, unless otherwise stated, were purchased from Sigma-Aldrich.

**Preparation of MPH and G-MPH**

The strain of *M. esculenta* (ACCC 50537) used in this study was purchased from the Agricultural Culture Collection of China. Morchella protein was produced by alkaline extraction followed by acidic precipitation from Morchella mycelium obtained by liquid fermentation according to our previously described method [Zhang et al., 2018c]. Briefly, the Morchella mycelia were homogenized and the proteins were extracted with NaOH solution (pH 12.0) by incubating in a water bath at 45°C for 1 h. The solution was centrifuged at 4,000×g for 20 min, the supernatant was adjusted to pH 4.1 with 2 mol/L HCl, and further centrifuged at 4,000×g for 20 min. After removing the supernatant, the precipitate was lyophilized to obtain the Morchella protein.

MPH and G-MPH were prepared based on our previous report [Zhang et al., 2018b]. Briefly, the Morchella protein was hydrolyzed using papain at an enzyme/protein ratio of 2% in a shaking water bath at 45°C for 3 h, at pH 6.0. Subsequently, the enzyme was inactivated in a boiling water bath for 10 min, followed by centrifugation at 4,000×g for 15 min. The supernatant was lyophilized to obtain MPH. G-MPH was produced by the Maillard reaction. MPH was mixed with xylose at a mass ratio of 1:3.7 in distilled water, adjusted to pH 11.8, and incubated in a boiling water bath for 60 min. The resulting solution was dialyzed (molecular weight cut-off, 200 Da) against distilled water for 48 h to remove unreacted xylose and the retentate was freeze-dried to obtain G-MPH. The MPH and G-MPH have been preliminarily characterized by infrared spectroscopy, fluorescence spectroscopy, and scanning electron microscopy in our previous study [Zhang et al., 2018a].

**Cell culture and treatment**

Caco-2 cells were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Caco-2 cells were cultured in DMEM medium containing 10% FBS and 1% streptomycin/penicillin at 37°C in a humidified 5% CO$_2$ incubator. To explore the protective effects of MPH and G-MPH against H$_2$O$_2$-induced oxidative stress, cells were grouped as follows: control group (Caco-2 cells without any treatment), model group (Caco-2 cells treated with 300 μM H$_2$O$_2$ for 6 h), MPH group (Caco-2 cells pretreated with 250 μg/mL MPH for 1 h followed by incubation with 300 μM H$_2$O$_2$ for 6 h), G-MPH group (Caco-2 cells pretreated with 250 μg/mL G-MPH for 1 h followed by incubation with 300 μM H$_2$O$_2$ for 6 h), and Vc group (Caco-2 cells pretreated with 250 μg/mL Vc for 1 h followed by incubation with 300 μM H$_2$O$_2$ for 6 h).

**Cell viability assay**

Cell viability was determined by 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, Caco-2 cells (1×10$^4$/mL) were seeded in 96-well plates (100 μL culture media/well). The cultures were maintained at 37°C for 24 h in a humidified 5% CO$_2$ incubator, and treated with specified concentrations of ligands for designated time. After the treatment, 20 μL of MTT (5 mg/mL) solution was added to each well and incubated for 3 h at 37°C. Thereafter, the supernatant was discarded from the wells, washed with phosphate-buffered saline (PBS; 1×, pH 7.4), and 150 μL of dimethyl sulfoxide (DMSO) was added to each well. The plates were placed on a shaker for 10 min and the absorbance was measured at 490 nm with an ELx800 microplate reader (Bio-Tek, USA).

**Measurement of intracellular ROS**

Intracellular ROS levels were detected using 2′,7′-dichlorofluorescein diacetate (DCFH-DA), a cell permeable non-fluorescent probe. After experimental treatment, the cells were
washed once with PBS, harvested, incubated with 10 μM DCFH-DA (diluted with serum-free culture media) at 37°C for 20 min, and washed three times with a serum-free cell culture solution. The fluorescence was measured by flow cytometry at λex=488 nm and λem=530 nm.

**Determination of MDA and GSH contents, T-AOC, and SOD and CAT activities**

After the experimental treatment, cells were washed once with PBS, harvested, lysed in PBS, and centrifuged at 13,000×g for 10 min at 4°C. The supernatant was used for subsequent measurements. Total protein was quantified using a commercial kit according to manufacturer’s instructions. Each experiment was repeated 3 times. MDA and GSH were detected using the manufacturer’s instructions. Each experiment was repeated 3 times. MDA and GSH are expressed as μmol/g protein and T-AOC, SOD, and CAT as U/mg protein.

**Immunofluorescence staining**

After the experimental treatment, the culture media were carefully discarded; the cells were washed three times with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. Cells were then washed with PBS (3 washes of 10 min each) and endogenous peroxidases were blocked with 3% H2O2-methanol solution for 10 min. Cells were then washed with PBS (3 washes of 10 min each), blocked with 10% goat serum for 20 min, followed by incubating with primary anti-Nrf2 antibody (1:100) at 37°C for 2 h. Cells were then washed with PBS (3 washes of 10 min each) and incubated with fluorescein isothiocyanate (FITC) conjugated secondary antibody (1:200 dilution) at 37°C for 1 h. After washing 3 times with PBS, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 5 min in the dark. Stained cells were washed with PBS (3 washes of 10 min each) and blotted to remove any residual liquid. Cells were mounted onto glass slides with antifade mounting medium and were analyzed using an inverted fluorescence microscope (Olympus IX51, Japan).

**TUNEL assay**

Apoptotic cells were detected using TUNEL assay kit following the manufacturer’s protocol. Briefly, after the experimental treatment, the cells were fixed in 4% paraformaldehyde for 30 min and washed three times with PBS. The cells were permeabilized with 1% Triton X-100 for 15 min at room temperature and rinsed three times with PBS. The cells were then incubated with 100 μL of TUNEL reaction mixture at 37°C for 60 min, rinsed three times with PBS, and stained with 100 μL of DAPI solution at 37°C in the dark for 5 min. The apoptotic cells were observed under an inverted fluorescence microscope (Olympus IX51, Japan).

**Annexin V-FITC/PI double staining assay**

Apoptotic cells were quantified using the Annexin V-FITC/PI apoptosis detection kit. After the experimental treatment, cells were harvested by trypsinization and washed twice with PBS. Then, the cells were collected by centrifugation at 1000×g for 5 min and approximately 3×10^6 cells were resuspended in 500 μL of binding buffer. They were then incubated with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) at room temperature for 15 min in the dark prior to flow cytometry (Becton Dickinson FACS Calibur).

**Measurement of mitochondrial transmembrane potential (MMP)**

JC-1 assay kit was used to detect MMP according to manufacturer’s instructions. Briefly, Caco-2 cells from different treatments were rinsed with 1× incubation buffer and incubated with 0.5 mL of JC-1 working solution at 37°C for 20 min in the dark. After rinsing twice with 1× incubation buffer, the cells were resuspended in 0.5 mL of 1× incubation buffer and the cell fluorescence was detected with a flow cytometer (Becton Dickinson FACS Calibur) (λex=488 nm, λem=530 nm). Data analysis was performed using CellQuest software (Becton-Dickinson).

**Western blot analysis**

Total proteins from Caco-2 cells were extracted with a total protein extraction kit and quantified using the BCA protein assay kit. After separation by SDS-PAGE (12%), proteins were transferred onto nitrocellulose (NC) membrane at 300 mA for 90 min, saturated with a blocking solution containing 5% non-fat milk for 1.5 h, and incubated with appropriate primary antibodies overnight at 4°C. The membranes were washed three times (10 min each) with TBST (Tris-buffered saline and Tween) and incubated with secondary antibody for 1.5 h at room temperature. The protein bands were visualized on a gel imaging system (Syngene G: BOXChemXRS, Cambridge, UK) using an ECL kit. The relative protein expression levels were quantified by the Gel-Pro32 software (MediaCybernetics Inc., USA) and normalized with GAPDH.

**Quantitative real-time PCR (qPCR) analysis**

Total RNA was extracted from the treated Caco-2 cells using Trizol reagent (Invitrogen, USA) according to the manufacturer’s protocol. RNA concentration was measured and quality was detected by measuring the absorbance at 260 and 280 nm using a UV-vis spectrophotometer (Shimadzu UV-2450). cDNA was synthesized in a 20-μL reaction volume from 2 μg of total RNA using the Reverse Transcription kit (K1622; Thermo Fisher Scientific). The qPCR reactions were performed with SYBR® Premix Ex TaqTM II (TaKaRa, Dalian, China) on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Primer sequences were as follows: Nrf2, F: 5′-CAACTACTCCCGTGTGCC-3′, R: 5′-AGTGAAGACGATGCAG-3′; NQO1, F: 5′-GCTGGCCATGATGCAAAGAC-3′, R: 5′-CATGCATCCCCTGAGATCC-3′; HO-1, F: 5′-CTCCACACTTCTCTTCC-3′, R: 5′-CTCCACACTTCTCTTCC-3′; GAPDH, F: 5′-TGCATGGAAGGTTCCCTCTC-3′, and R: 5′-TGCATGGAAGGTTCCCTCT-3′. The gene expression data were normalized to the housekeeping gene (GAPDH) and expressed as 2^{-ΔΔCT} values.

**Statistical analysis**

Data are represented as mean ± SD of triplicate experiments. Data were analyzed by one-way ANOVA using
SPSS 19.0. The differences between groups were performed by Duncan’s multiple range test and considered statistically significant at $p<0.05$.

**RESULTS AND DISCUSSION**

**MPH and G-MPH protected Caco-2 cells from H$_2$O$_2$-induced injury**

To examine the cytotoxic potential of MPH, G-MPH, and H$_2$O$_2$, cell viability of Caco-2 cells under different treatments was measured by MTT assay. MPH and G-MPH had no negative effects at the tested concentrations (62.5–250 μg/mL) (Figure 1A). However, H$_2$O$_2$ (100–500 μM) significantly ($p<0.05$) decreased cell viability after incubating for 6 h in a concentration-dependent manner (Figure 1B). At a concentration of 300 μM, H$_2$O$_2$ moderately decreased cell viability (52.58%). Therefore, the cells were treated with 300 μM H$_2$O$_2$ for 6 h in the following experiments.

Next, we evaluated the protective effects of MPH and G-MPH against H$_2$O$_2$-induced oxidative injury. As shown in Figure 1C, exposure of Caco-2 cells to 300 μM H$_2$O$_2$ for 6 h decreased cell viability by 47.31% compared to the control group, whereas pretreatment with MPH and G-MPH both significantly ameliorated the decrease in cell viability ($p<0.05$). G-MPH showed significantly more protective effect on cell proliferation than MPH ($p<0.05$). The protective effect of 500 μg/mL G-MPH was almost the same as that of 250 μg/mL Vc. This result implies that MPH and G-MPH can attenuate H$_2$O$_2$-induced cytotoxicity and exhibit significant protective effects against H$_2$O$_2$-induced oxidative injury. Furthermore, both MPH and G-MPH at 250 and 500 μg/mL concentrations caused similar protective effects. Therefore, 250 μg/mL was selected as the optimal concentration (MPH and G-MPH) in H$_2$O$_2$-treated Caco-2 cells for subsequent experiments. Similar study was conducted by Zha et al. [2015] who reported that neither the shrimp by-product protein hydrolysate nor its glycosylated derivative had any cytotoxic effect at 1000 μg/mL Vc. This result implies that MPH and G-MPH can attenuate H$_2$O$_2$-induced cytotoxicity and exhibit significant protective effects against H$_2$O$_2$-induced oxidative injury.

**MPH and G-MPH inhibited H$_2$O$_2$-induced ROS generation and lipid peroxidation in Caco-2 cells**

Growing evidences support that oxidative damage plays a crucial role in the pathophysiology of various diseases [Fer-
When the body is under oxidative stress, there is a shift in the redox balance between oxidants and antioxidants, resulting in an increase in the amount of ROS in the cells [Mariani et al., 2005]. Excessive ROS can cause oxidation of proteins and lipids in cells, destroying the integrity of nuclear DNA and mitochondria, and ultimately leading to cell death [Yamaguchi et al., 2015]. The levels of ROS in Caco-2 cells were detected by DCFH-DA probe combined with flow cytometry. As shown in Figure 2A to B, H₂O₂ treatment notably increased production of ROS in Caco-2 cells compared to control. However, pretreatment with MPH, G-MPH, or Vc significantly reduced H₂O₂-induced ROS generation and the ROS levels in Caco-2 cells reduced to 30.92±0.71%, 15.18±0.29%, and 4.12±0.09%, respectively, which were significantly lower than that in the model group (p<0.05).

Lipid peroxidation is one of the major events in free radical-induced cell oxidative damage. MDA, a major by-product of membrane lipid peroxidation, is considered as a biomarker of cell membrane injury. MDA can further amplify the effect of ROS causing a cascade of chain reactions, destroying biological macromolecules such as nucleic acids and proteins, and causing various diseases in the body [Je & Lee, 2015; Lee et al., 2004]. The effects of MPH and G-MPH on intracellular MDA levels are presented in Figure 2C. Along with ROS generation, MDA level in model group was also significantly more than that in the control group (p<0.05). Pretreatment with MPH, G-MPH, or Vc significantly attenuated H₂O₂-induced MDA production (p<0.05). Collectively, these results reveal that MPH and G-MPH may prevent the formation of ROS, inhibit lipid peroxidation, and accordingly protect the cells from H₂O₂-induced oxidative damage. These findings are similar to the earlier study which reported the antioxidative activities of rice dreg protein hydrolysate [Zhang et al., 2016]. The protective effect may be due to the wide range of antioxidant activities MPH and G-MPH possess and the roles they play as H₂O₂ and free radical scavengers, which have been confirmed in our previous studies [Zhang et al., 2018b].

**FIGURE 2.** Effects of MPH and G-MPH on ROS generation and lipid peroxidation by H₂O₂ in Caco-2 cells. (A) and (B) ROS identified using DCFH-DA staining combined with flow cytometry and the corresponding data quantified. (C) Lipid peroxidation assessed by MDA assay. Data are means ± SD from 3 independent experiments. *p<0.05 versus control, *p<0.05 versus model. MPH – Morchella protein hydrolysate; G-MPH – glycosylated derivative of MPH.
sumed by this defense system, which in turn protect cells from damage and maintain a stable state [Xu et al., 2016]. GSH, T-AOC, and antioxidant enzymes such as SOD and CAT play an extremely important role in the antioxidant defense system of cells. Therefore, these components were evaluated to investigate whether the protective effects of MPH and G-MPH were associated with an improvement in antioxidant defense capacity of Caco-2 cells. After exposure to 300 μM H_2O_2 for 6 h, GSH level, T-AOC, and SOD and CAT activities were markedly decreased by 47.72%, 80.23%, 76.12%, and 66.57%, respectively, as compared to the control group (p<0.05) (Figure 3). As expected, compared with the model group, pretreatment of the cells with MPH and G-MPH significantly attenuated the decrease in the levels/activities of these components in H_2O_2-treated cells. Particularly, pretreatment of the cells with G-MPH reversed the H_2O_2-induced decrease in GSH levels by 1.44 folds (Figure 3A), T-AOC by 3.03 folds (Figure 3B), SOD activity by 3.44 folds (Figure 3C), and CAT activity by 2.28 folds (Figure 3D). However, the effects were not comparable to Ve. These data collectively indicate that MPH and G-MPH can exert their protective effects by improving the antioxidant defense capacity of Caco-2 cells. Results of the current study were similar to those of Shi et al. [2014], who found that eggshell membrane peptides protect Caco-2 cells from H_2O_2-induced oxidative damage by improving antioxidant enzyme activity and glutathione synthesis.

**Protective effects of MPH and G-MPH against oxidative injury involving activation of Nrf2 signaling pathway**

The Nrf2-antioxidant response element signaling pathway is a main endogenous antioxidant stress pathway that plays a key role in enhancing the antioxidant defense system of cells [Nguyen et al., 2009]. Nrf2, the main component of the signaling pathway, is normally located in the cytoplasm and combines with its cytosolic inhibitor, Keap-1. Various stimuli including oxidants, electrophiles, certain disease processes, and exogenous small (natural) molecules can activate Nrf2 through its disassociation from Keap-1. Activated Nrf2 translocates into the nucleus where it regulates the gene expression of phase II detoxifying and antioxidant enzymes to protect the organism from oxidative stress.
Cytoprotective Effect of *Morchella esculenta* and related injuries [Chang *et al.*, 2018; Zhang *et al.*, 2018a]. Activation of Nrf2 is considered as a therapeutic target for neurodegenerative and cardiovascular diseases [Cuadrado *et al.*, 2009; Li *et al.*, 2009].

To further elucidate the mechanisms underlying the cytoprotective effects of MPH and G-MPH, the impact on Nrf2 signaling pathway was investigated. The Nrf2 nuclear translocation detected by immunofluorescence (Figure 4A) showed that Nrf2 was mainly present in the cytoplasm of normal cells. H$_2$O$_2$-induced oxidative stress promoted partial translocation of Nrf2 into the nucleus, while pretreatment with MPH, G-MPH, or Vc significantly promoted the nuclear translocation of Nrf2. This result was coincident with those reported by Yang *et al.* [2017], who demonstrated that glycosylated fish protein hydrolysates induced nuclear translocation of Nrf2 to activate the Nrf2 signaling pathway.
The effects of MPH and G-MPH on protein and mRNA expression of several components related to Nrf2 signaling pathway including Nrf2, NQO1, and HO-1 were evaluated by Western blotting and qPCR, respectively. As shown in Figure 4B to C, the expressions of Nrf2, NQO1, and HO-1 at both protein and mRNA levels were significantly higher in the model group than in the control group after H$_2$O$_2$-induced oxidative damage ($p<0.05$). This indicates that H$_2$O$_2$ activates Nrf2 antioxidant pathway while inducing oxidative stress. Compared with the model group, pretreatment with MPH, G-MPH, or Vc further increased the protein and mRNA expression levels of Nrf2, NQO1, and HO-1 in the H$_2$O$_2$-treated Caco-2 cells. These data suggest that MPH and G-MPH may activate the Nrf2 signaling pathway which might serve as a crucial mechanism for MAP and G-MAP to exert their protective effects on cell oxidative stress. Similarly, Pyo et al. [2016] reported that glycosylated whey protein concentrate activated the Nrf2-dependent pathway and induced expressions of antioxidant enzymes and phase II enzymes that had cytoprotective effects against oxidative injury in HepG2 cells. 

**MPH and G-MPH ameliorated H$_2$O$_2$-induced apoptosis via restoring MMP and regulating apoptosis-related protein expression in Caco-2 cells**

Apoptosis is a mechanism which enables eukaryotic organisms to eliminate unwanted or defective cells through an orderly process of cell decomposition that is essential for the development of normal tissues [Li et al., 2016]. To maintain tissue homeostasis, a proper regulation of cell proliferation and apoptosis is critical [Pan et al. 2018]. To determine whether the protective effects of MPH and G-MPH against H$_2$O$_2$-induced injury were by counteracting apoptosis, the TUNEL staining and Annexin V/PI assay were performed to detect the apoptotic cells. TUNEL staining revealed that the number of TUNEL positive cells in the model group was notably increased in comparison with the control group. However, pretreatment with MPH, G-MPH, or Vc markedly decreased the number of TUNEL positive cells when compared with the model group (Figure 5A). At the same time, Annexin V/PI assay showed that the apoptosis rate was higher in the model group than that in the control group (42.78±0.68% versus 5.88±0.19%). Moreover, when compared with model group, pretreatment with MPH, G-MPH,
or Vc significantly decreased the apoptosis rate to 30.92±0.71, 14.74±0.21, and 11.37±0.51%, respectively (Figure 5B). Apparently, these results suggest that MPH and G-MPH can exert potential protective effects against H₂O₂ injury through the inhibition of apoptosis.

Mitochondria play a crucial role in the process of cell apoptosis. The loss of MMP is regarded as an important signal for damage to mitochondrial structure. Therefore, we assessed whether MPH and G-MPH exerted anti-apoptotic effects by restoring MMP in Caco-2 cells using JC-1 assay. As shown in Figure 6A to B, a marked increase in the number of cells with low MMP was observed in the model group compared with the control group; however, the number of cells with low MMP was significantly decreased on pretreatment with MPH, G-MPH, or Vc (30.90±0.63% for MPH, 14.30±0.28% for G-MPH, and 12.11±0.95% for Vc) compared with the model group (p<0.05). This indicated that MPH and G-MPH can restore MMP loss triggered by H₂O₂, stabilize mitochondrial function, and accordingly attenuate H₂O₂-induced apoptosis.

Apoptosis is regulated by the pro-apoptotic Bax and anti-apoptotic Bcl-2 proteins [Hu et al., 2015]. Increased Bax/Bcl-2 ratio (i.e., increased expression of Bax and reduced expression of Bcl-2) is considered as a reliable indicator of apoptosis [Xue et al., 2014]. In addition, Caspase-3 is the primary executor of apoptosis. Activation of caspase-3 triggers DNA fragmentation and chromatin condensation leading to an irreversible cascade of events progressing towards cell death [Liu et al., 2007; Neuzil et al., 2004]. To further understand the mechanisms via which MPH and G-MPH regulate cell apoptosis, the expression levels of apoptosis-related proteins including Bax, Bcl-2, and caspase-3 were investigated using Western blot analysis. Figure 6C shows the expression levels of proteins Bax, Bcl-2, and caspase-3 in each group presented by Western blots using GAPDH as an internal control. The corresponding quantitative results showed that the levels of Bax and caspase-3 were evidently increased, whereas the expression of Bcl-2 was significantly reduced in the model group after H₂O₂ treatment when compared with the control group (p<0.05). However, pretreatment with MPH, G-MPH, or Vc remarkably inhibited Bax and caspase-3 expression and elevated Bcl-2 expression in comparison with the model group (p<0.05) (Figure 6D). Thus, it suggests that the anti-apoptotic effects of MPH and G-MPH are associated with
the regulation of Bax, Bcl-2, and caspase-3 protein expressions. As well, Jiao et al. [2018] confirmed that the cocaine- and amphetamine-regulated transcript (CART) peptide can decrease the expression of Bax and caspase-3 and increase the expression of Bcl-2 to inhibit neuronal apoptosis while attenuating oxidative injury in rat hippocampal neurons.

CONCLUSIONS

In the present study, the cytoprotective effects of MPH and G-MPH against oxidative stress and their underlying mechanisms were assessed in Caco-2 cells. MPH and G-MPH attenuated H₂O₂-induced cytotoxicity in Caco-2 cells and exhibited protective effects by inhibiting the productions of ROS and MDA, enhancing antioxidant defense capacity, and activating Nrf2 signaling pathway under H₂O₂-induced oxidative stress. Moreover, MPH and G-MPH prevented H₂O₂-induced apoptosis by restoring the loss of MMP and regulating the expression of apoptosis-related proteins. Collectively, MPH and G-MPH can protect Caco-2 cells against H₂O₂-induced oxidative injury via elevating antioxidant response and inhibiting apoptosis. Therefore, the current research suggests that MPH and G-MPH can be excellent nutraceutical/functional food components or potential therapeutic agents to prevent or treat oxidative stress-induced diseases.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

REFERENCES


