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Hydrogen peroxide-induced oxidative stress and apoptosis in SH-SY5Y cells: Protective effect of *Momordica charantia* fruit extract

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Abstract

Oxidative stress triggers apoptosis in neuronal cells, resulting in cellular loss, which is critical in the pathogenesis of neurodegenerative diseases. *Momordica charantia* (MC), a traditional medicinal plant, is famous for its variety of health benefits, including its anti-diabetic, anti-inflammatory, and antioxidant properties. The purpose of this study was to investigate on how MC might affect oxidative stress and apoptosis caused by H_2O_2. First, we investigated whether ethanol extract of MC in the presence of H_2O_2 attenuated cell death in human neuroblastoma SH-SY5Y cells. MC improved H_2O_2-stimulated intracellular reactive oxygen species (ROS) production. Additionally, MC reduced caspase activation, which greatly improved cell viability and avoided H_2O_2-induced apoptosis. Through its anti-oxidant and anti-apoptotic properties, MC ethanol extract has been shown to be effective in protecting against H_2O_2-induced cell death.

Keywords: Hydrogen peroxide, Oxidative stress, Apoptosis, *Momordica Charantia*

1. Introduction

Neurodegenerative diseases are typically associated with cytoplasmic protein aggregation in neurons caused by oxidative stress and are characterized by apoptosis-induced progressive cell loss in specific vulnerable neuronal cells (1). Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) are neurodegenerative diseases with clinical findings such as progressive cognitive loss (dementia) and motor impairment (ataxia). The quality of life and longevity of elderly people are both impacted by these cognitive and motor impairments (2). It is critical to develop effective treatment strategies for neurodegenerative diseases that affect the elderly population. Therefore, antioxidants which protect neuronal cells from oxidative stress-induced apoptosis could potentially be used as therapeutic agents for neurodegenerative diseases.

Because of their rich antioxidant content, natural products have long been used as traditional medicines for the treatment of neurodegenerative diseases (3). Many studies conducted over the last several decades have demonstrated the protective effects of polyphenolic compounds extracted from natural products against neuronal cell damage caused by oxidative stress (4,5). Bioactive polyphenolic antioxidants have neuromodulatory properties, activating various intracellular signaling pathways that are important for neuroprotection. The regulation of the mitochondrial apoptosis cascade, which is finely tuned by the imbalance of apoptotic and anti-apoptotic proteins, is the molecular mechanism of neuroprotection. Neuroprotection mediated by antioxidant polyphenols can be achieved by increasing the expression of the anti-apoptotic Bcl-2, thus preventing apoptosis (6). Another neuroprotective mechanism is the suppression of caspases in controlling oxidative stress-mediated apoptosis, which is significant in the pathogenesis of neurodegenerative disorders (7).

Momordica charantia (MC), known as bitter melon or bitter gourd with its rich polyphenol content, is widely grown and generally consumed as an important medicinal plant in Asian countries (8). MC contains several bioactive compounds: triterpene, protein, steroids, alkaloids, inorganic, lipid, and phenolic compounds (9). Recent studies have reported the anti-bacterial (10), anti-oxidant (11), anti-inflammatory (12), and anti-diabetic (13) effects of various MC extracts.

The aim of this study was to determine the contribution of MC to the control of H_2O_2-induced oxidative stress and apoptosis. In our study, we report that MC has biological activities to reduce H_2O_2-induced cell death and cellular ROS production. We also demonstrated that MC decreased caspase-3 and caspase-9 expressions, inhibiting the process of H_2O_2-induced apoptosis in SH-SYY cells.

2. Materials and Methods

2.1. 2.1. Preparation *Momordica Charantia* Ethanol Extract

Fresh plant material (Ripe fruit without seeds) weighing 350 grams was dried under the shade. The plant parts were fully

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dried, then ground and the 31,7 g of the powdered plant was macerated in 70% ethanol for 72 hours. The extract was filtered using filter paper, the filtrate evaporated using a rotary evaporator, and then crude extract was obtained in a lyophilizer.

2.2. Cell Culture

SH-SY5Y (Human neuroblastoma) cells were cultured using DMEM supplemented with 10% heat-inactivated FBS and 0.1% penicillin/streptomycin in a 75 cm^2 culture flask at 37 °C with 5% CO2 humidified atmosphere. Every 2-3 days, the medium was changed, and cells were subcultured once they had reached 80–90% confluency. Cells were collected by centrifugation at 1000 rpm for five minutes after being digested with 0.25% trypsin and then resuspended in new media. In suitable assay plates, cells were seeded, and they grow overnight. For further research, adherent cells were used.

2.3. Cytotoxicity assay

For cytotoxicity analysis, the MTT reduction assay was performed (14). In 96-well plates with 2% FBS media, SH-SY5Y cells were seeded at a density of $1{,}5x10^3$ cells/well for the MTT assay. The cells were serum-starved before extract or H_2O_2 treatment for 24 hours. H_2O_2 was incubated for 24 hours in the presence of the extract in the study, where we demonstrated the protective action of the extract against the H_2O_2 cytotoxicity. Following that, the cells were treated with MTT (0.5 mg/mL) for an additional 2–3 hours. Each well's medium was removed and then DMSO was added to dissolve the purple formazan crystals and the absorbance of each well's solution was then determined using a microplate reader at 570 nm.

2.4. Measurement of Intracellular ROS

H_2DCF-DA Reagent was used to measure intracellular ROS production (15). Cells were initially exposed to H_2O_2 at various concentrations (0-800 µM) for 1 hour to determine the concentration at which H_2O_2 stimulated intracellular ROS generation. However, to evaluate the ROS scavenging ability of the extract, 800 µm H_2O_2 was applied to the cells in the presence of various concentrations of the extract for 1 hour. Subsequently, cells were incubated in DMEM (without phenol red) with H_2DCF-DA Reagent (25 µM) for 30 minutes in the dark. The change in fluorescence intensity was detected by fluorescence spectroscopy with excitation/emission at 485 nm / 535 nm.

2.5. Western Blot Analysis

The standard procedure for Western blotting was followed (16). Cells were treated with H_2O_2 (150 µM) alone or in combination with varying concentrations of extract for 3 hours. Following the treatment procedure, cells were lysed in RIPA buffer, and the protein content was determined using a BCA protein assay kit in accordance with the manufacturer's instructions. Proteins were separated on polyacrylamide gels with a 10% concentration before being transferred to the PVDF membrane. The membranes were blocked using a non-fat milk solution for 1 hour, and then they were treated with specific primary antibodies overnight. The next day, after the primary antibody was removed, membranes were incubated with the matched secondary antibody for 4 hours. ECL reagent was used to make the bands visible, and Image J software was used to measure the bands' intensity.

2.6. Acridine orange/Ethidium bromide (AO/EB) staining

Acridine orange/ethidium bromide (AO/EB) double staining was used to examine apoptotic and necrotic morphological changes in cells under fluorescence microscopy (17). 150 µM H_2O_2-exposed cells in combination with or without varying concentrations of extract for 3 hours were collected and washed three times in phosphate-buffered saline (PBS). Following centrifugation at 1000 rpm, the pellets were gently resuspended in 20 µL of medium containing 2.5 µL of dye solution (100µg/mL in equal concentration, AO/EB in DMEM w/o Phenol red). Each image was captured with excitation at 488 nm and emission at 520 nm on fluorescence microscope slides coated with a total of 10 µL of the cell-dye combination. Three or more random images were acquired for each well. At least 200 cells were counted on each slide during the triplicate testing.

2.7. Statistical data analysis

The data was analyzed using the "Student's t test" from the SigmaPlot 12.0 package program. $p<0.05$ was regarded as statistically significant.

3. Results

3.1. *Momordica charantia* extract protected SH-SY5Y against H_2O_2-Induced Cell Cytotoxicity

The cell viability test was utilized to assess the viability of SH-SY5Y cells after exposure to various concentrations of *Momordica charantia* extract and to establish whether nontoxic concentrations of *Momordica charantia* may alleviate the toxicity of H_2O_2 against SH-SY5Y cells. First, we investigated how varied H_2O_2 and extract concentrations affected the viability of cells. Fig. 1A shows the findings of the cell viability percentage following treatment with various concentration of H_2O_2 and extract. The viability of SH-SY5Y cells was reduced to 80.08% and 52.87%, respectively, when treated with 50 µM and 75 µM of H_2O_2, compared to 100% in the control group. However, up to a concentration of 15 µg/ml, *Momordica charantia* extract showed no evidence of cytotoxicity (Fig. 1B).

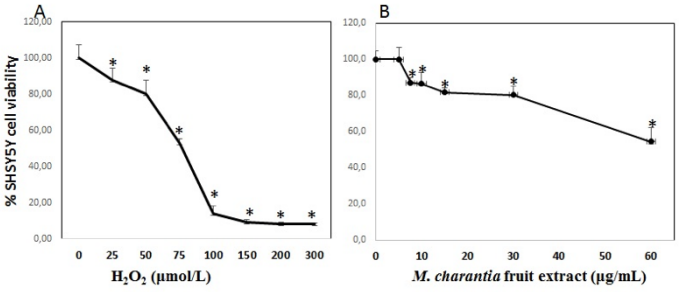

Fig. 1. A. The effects of H_2O_2 on the viability of SH-SY5Y cells. Cells were treated with indicated concentration of H_2O_2 for 24h. **B.** The effects of Momordica charantia fruit extract on the viability of

SHSY5Y cells. Cells were treated with a series of concentration of extract for 24 h. Data are expressed as mean ± SD of five independent experiments (n = 5). *p<0,05 vs. Control cell

As a result, the nontoxic concentrations of 5.0, 7.5, 10.0, and 15.0 µg/ml were selected for further tests to assess the protective effect of *Momordica Charantia* extract. However, *Momordica Charantia* extract treatment (at concentrations of 5.0, 7.5, 10.0, and 15.0 µg/ml) in the presence of H_2O_2 (50 µM and 75 µM) significantly inhibited in a concentration-dependent manner the cytotoxicity induced by H_2O_2 in SH-SY5Y cells vs. H_2O_2 treated cell. (Fig. 2).

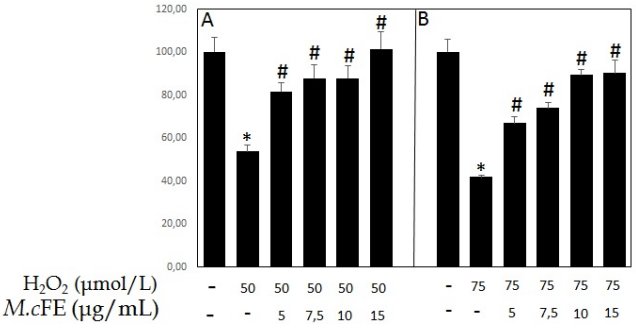

Fig. 2. Protective effects of Momordica charantia fruit extract on SHSY5Y cells against H_2O_2 -induced cell injury. Cells were treated with extract (5, 7,5, 10 and 15 µg/mL) in the presence of 50 and 75 µM H_2O_2 for 24 h. Data are expressed as mean ± SD of five independent experiments (n = 5). *p<0,05 *vs.* Control cell, #p<0,05

3.2. Effect of *Momordica charantia* extract on ROS production in H_2O_2-induced SH-SY5Y cells

The next step was to test the ROS production in cells using the H_2DCFDA reagent, a fluorescent dye that shows ROS, in order to see whether *Momordica charantia* extract might alleviate the oxidative stress caused by H_2O_2-induced ROS production. As can be shown in Fig. 3A, the H_2O_2-treated (0-800 µM) cells considerably raised the intensity of the DCF-liberated fluorescent signal in a dose-dependent manner, and the signal was markedly reduced in the presence of *Momordica charantia* extract. In the presence of Momordica charantia extract and H_2O_2 (800 µM) in the medium, DCF-liberated fluorescent signal decreased with increasing extract concentration (5.0, 7.5, 10.0, and 15.0 µg/ml), suggesting the H_2O_2 scavenging effect of the extract (Fig. 3B)

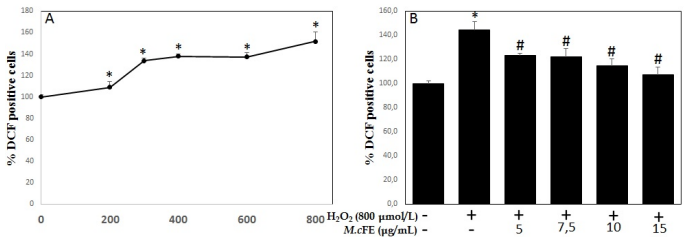

Fig. 3. A. The effects of H_2O_2 on ROS production in SHSY5Y cells. Cells were treated with indicated concentration of H_2O_2 for 1h. **B.** Effects of Momordica charantia fruit extract on intracellular ROS production. Cells were treated with extract (5, 7.5, 10 and 15 µg/mL) in the presence of 800 µM H_2O_2 for 1h. Data are expressed as mean ± SD of five independent experiments (n = 5). *p<0,05 *vs.* Control cell, #p<0,05 *vs.* H_2O_2 treated cell

3.3. H_2O_2 promotes caspase-3 and caspase-9 expression in SH-SY5Y cells: Effect of *Momordica charantia* extract

Apoptosis-associated protein (caspase-9 and caspase-3) levels were assessed to validate *Momordica charantia* extract protection against H_2O_2-induced apoptosis. Compared to H_2O_2 treatment alone, Western blot analysis showed that treatment with *Momordica charantia* extract reduced the expression of caspase-9 and caspase-3 (Figure 4).

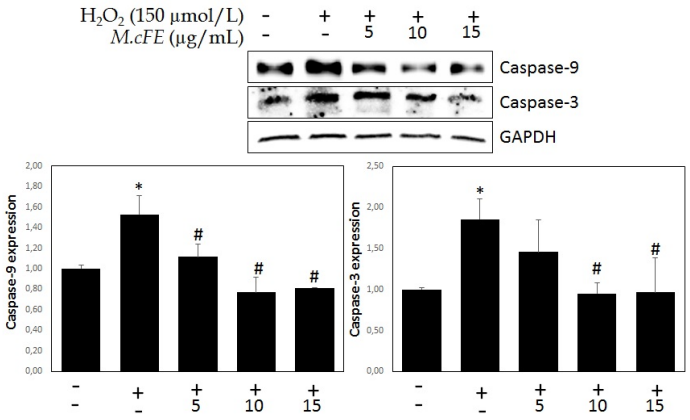

Fig. 4. Representative blots showing caspase 9 and caspase 3 expression. Normalized values of caspase 9 and caspase 3. Data are expressed as mean ± SD of five independent experiments (n = 3). *p<0,05 *vs.* Control cell, #p<0,05 *vs.* H_2O_2 treated cell.

3.4. H_2O_2 increased proportion of apoptotic and necrotic cells: Effect of *Momordica charantia* extract

Four cell phases upon exposure to H_2O_2-induced stress were detected using AO/EB double staining: While the nuclei of both live and dead cells are stained green by acridine orange (AO), only cells that have lost membrane integrity are stained red by ethidium bromide (EB). Consequently, whereas early apoptotic cells have fragmented nuclei that are brilliant green in color, living cells will appear to be uniformly green. Condensed and fragmented orange chromatin is a sign of late apoptosis in cells. The percentage of necrotic and apoptotic cells considerably increased after exposure to H_2O_2. Extract incubation in the presence of H_2O_2 partly but significantly enhanced the proportion of surviving cells and inhibited necrosis and apoptosis-related cell death (Fig. 5).

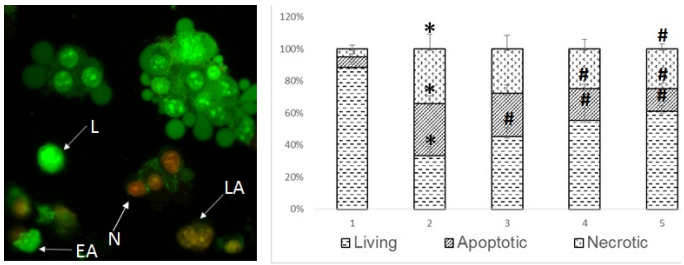

Fig. 5. Acridine Orange/Ethidium Bromide (AO/EB) dual staining of SHSY5Y cells. Bar 1: Control cell, Bar 2: H_2O_2 (150 µM) treated cell, Bar 3: Effect of McFE (5 µg/mL) at the presence of H_2O_2, Bar 4: Effect of McFE (10 µg/mL) at the presence of H_2O_2 Bar 5: Effect of McFE (15 µg/mL) at the presence of H_2O_2

4. Discussion

One of the main contributing reasons to neurodegeneration is the excessive creation of ROS, which results in oxidative damage to proteins, lipids, and DNA (18). A significant contributor to the production of intracellular ROS is the mitochondrial electron transport system, and the mitochondria are necessary for the process of ROS-mediated cell death (19). Furthermore, H_2O_2 immediately causes mitochondrial dysfunction, followed by an early release of intracellular ROS, which results in an immediate depolarization of the inner mitochondrial membrane (20). This procedure most likely accelerates the disruption of the mitochondrial membrane potential and the release of apoptosis-inducing compounds that activate caspase-dependent signaling cascades to trigger apoptosis (21). For the prevention and treatment of neurodegenerative diseases, it is therefore essential to find functional foods or bioactive substances that act against oxidative stress. It was determined in this study whether *Momordica charantia* extract inhibits H_2O_2-induced oxidative stress and apoptosis in SH-SY5Y cells. The results showed that H_2O_2 treatment caused intracellular ROS accumulation as well as apoptosis by preventing cell survival in SH-SY5Y cells. However, when SH-SY5Y cells were simultaneously exposed to H_2O_2 and *Momordica charantia*, H_2O_2-induced ROS generation, cell viability reduction, and increased apoptotic cell death were significantly attenuated, as previously reported in research on other SK-N-MC neuroblastoma cells (22). Therefore, we presume that *Momordica charantia* protects neuronal cells by inhibiting the oxidative effect of H_2O_2 and thereby reducing H_2O_2-induced apoptosis. *Momordica charantia* has a neuroprotective effect not just found in this study. It was emphasized in the previous study that charantin isolated from *Momordica charantia* demonstrated neuroprotective effects in SH-SY5Y cells by preventing nerurotoxin MPP+ and tunicamycin induced neuronal damage and endoplasmic reticulum stress (23). Despite the neurotoxicity caused by polycyclic aromatic hydrocarbons, *Momordica charantia* was found to prevent cell death in rat hippocampus neuronal cells by regulating the cell cycle and MAPK cascade with its ROS scavenging function (24). In a different study, lyophilized *Momordica charantia* juice was shown to have anti-oxidative and neuroprotective properties in diabetic cerebral ischemia reperfusion injury (25). *Momordica charantia* was used to create exosome-like nanoparticles, and their neuroprotective effects on brain ischemia reperfusion injury were also investigated. This study demonstrated how exosome-like nanoparticles could quickly cross the blood-brain barrier and inhibit neuronal apoptosis by modulating the AKT/GSK-3B pathway (26).

In conclusion, Overall, increased oxidative stress, as well as subsequent apoptotic neuronal cell loss, is the mechanism underlying the pathogenesis of neurodegenerative diseases. No specific drug has been developed yet for the treatment of neurodegenerative diseases. In the context, research on the neuroprotective properties of antioxidant-rich herbal extracts and plant-based compounds in neurodegenerative diseases has gained popularity. The results of our present study clearly demonstrated that *Momordica charantia* fruit extract exerted a neuroprotective effect against H_2O_2-induced cell death, ROS production, and apoptosis in SH-SY5Y cells. Our results also confirmed that fruit extract from *Momordica charantia*, a type of antioxidant, may have neuroprotective properties.

Conflict of interest

The authors declare that they have no competing interests.

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Authors' contributions

Concept: A.C., H.D., Design: A.C., H.D., Data Collection or Processing: A.C., H.D., Analysis or Interpretation: A.C., H.D., Literature Search: A.C., H.D., Writing: A.C

Ethical Statement

Ethics committee approval is not required for this study.

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