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# Neuroprotective effects of hesperetin on $H_2O_2$-induced damage in neuroblastoma SH-SY5Y cells

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**Conflict of Interest**
The authors declare no potential conflicts of interests.

## ABSTRACT

**BACKGROUND/OBJECTIVES:** Oxidative stress is a fundamental neurodegenerative disease trigger that damages and decimates nerve cells. Neurodegenerative diseases are chronic central nervous system disorders that progress and result from neuronal degradation and loss. Recent studies have extensively focused on neurodegenerative disease treatment and prevention using dietary compounds. Heseperetin is an aglycone hesperidin form with various physiological activities, such as anti-inflammation, antioxidant, and antitumor. However, few studies have considered hesperetin's neuroprotective effects and mechanisms; thus, our study investigated this in hydrogen peroxide ($H_2O_2$)-treated SH-SY5Y cells.
**MATERIALS/METHODS:** SH-SY5Y cells were treated with $H_2O_2$ (400 μM) in hesperetin absence or presence (10–40 μM) for 24 h. Three-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assays detected cell viability, and 4′,6-diamidino-2-phenylindole staining allowed us to observe nuclear morphology changes such as chromatin condensation and apoptotic nuclei. Reactive oxygen species (ROS) detection assays measured intracellular ROS production; Griess reaction assays assessed nitric oxide (NO) production. Western blotting and quantitative polymerase chain reactions quantified corresponding mRNA and proteins.
**RESULTS:** Subsequent experiments utilized various non-toxic hesperetin concentrations, establishing that hesperetin notably decreased intracellular ROS and NO production in $H_2O_2$-treated SH-SY5Y cells ($P < 0.05$). Furthermore, hesperetin inhibited $H_2O_2$-induced inflammation-related gene expression, including interluekin-6, tumor necrosis factor-α, and nuclear factor kappa B (NF-κB) p65 activation. In addition, hesperetin inhibited NF-κB translocation into $H_2O_2$-treated SH-SY5Y cell nuclei and suppressed mitogen-activated protein kinase protein expression, an essential apoptotic cell death regulator. Various apoptosis hallmarks, including shrinkage and nuclear condensation in $H_2O_2$-treated cells, were suppressed dose-dependently. Additionally, hesperetin treatment down-regulated Bax/Bcl-2 expression ratios and activated AMP-activated protein kinase-mammalian target of rapamycin autophagy pathways.
**CONCLUSION:** These results substantiate that hesperetin activates autophagy and inhibits apoptosis and inflammation. Hesperetin is a potentially potent dietary agent that reduces neurodegenerative disease onset, progression, and prevention.

**Keywords:** Neurodegenerative disease; hydrogen peroxide; inflammation; apoptosis; hesperetin

**Author Contributions**

Conceptualization: Yun JM; Funding Acquisition: Yun JM; Investigation: Moon HR, Yun JM; Methodology: Moon HR, Yun JM; Visualization: Moon HR; Supervision: Yun JM; Writing - original draft: Moon HR; Writing - review & editing: Yun JM.

## INTRODUCTION

The brain demands high oxygen, low antioxidant enzymes, and catalytic transition metal abundance, making it more susceptible to oxidative damage [1]. Many studies demonstrate how oxidative stress instigates cell damage and neurodegenerative diseases [2]. Reactive oxygen species (ROS) generate oxidative stress, damaging cell membrane structures, proteins, lipids, and DNA functions that ultimately cause cell death and exacerbate neurodegenerative diseases [3]. Mitochondria frequently generate ROS hydrogen peroxide ($H_2O_2$), a common cellular oxidative stress [4,5]. $H_2O_2$ influences mitochondrial dysfunction and caspase activation, leading to DNA damage, inflammation, and apoptosis in the human body [6].

Recent studies reported that the blood and brain of neurodegenerative disease patients exhibited heightened inflammatory cytokine levels such as interleukin (IL)-6, tumor necrosis factor (TNF)-α, and cyclooxygenase-2 (COX-2) [7]. Hassanzadeh-Taheri *et al.* [8] conveyed that rosmarinic acid prevented IL-6 and TNF-α overproduction in mice with lipopolysaccharide (LPS)-induced cognitive impairment. In addition, Ha *et al.* [9] established that 6-Shogaol inhibited COX-2, inducible nitric oxide synthase (iNOS), and nuclear factor (NF)-κB in LPS-treated BV2 cells to protect neurons. Central nervous system damage activates neuroinflammation, associated with tissue repair, whereas chronic neuroinflammation relates to neurodegenerative disease progression. Neurodegenerative disease patients exhibit significant neuronal attenuation [10-12], corroborated by in vitro and in vivo studies, chromatin condensation, DNA fragmentation, and caspase activity, which are morphological apoptosis features [13]. Apoptosis is characterized by typical biochemical and morphological features, such as cell contraction, membrane-bound apoptotic body division, caspase activation, and increased apoptosis promoter [14]. ROS-induced apoptosis reduces mitochondrial caspases, B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), and Bcl-2 [15]. In addition, pro-apoptotic factor activation such as Bax, caspase, poly (ADP-ribose) polymerase (PARP), and their intracellular pathways result in cell death [16,17].

The mitogen-activated protein kinase (MAPK) signaling pathway is imperative for complex cellular programs such as differentiation, proliferation, transformation, and apoptosis; thus, this signal is a vital apoptotic cell death regulator. Oxidative stress activates the MAPK pathway, induces brain nerve cell death, and causes brain disease [17]. In addition, intracellular ROS generation kindles oxidative stress closely related to normal physiological homeostasis and disease [18].

The autophagy pathway eliminates abnormal cellular protein aggregates and regulates ROS and apoptosis levels [19,20]. ROS can transcriptionally and post-translationally regulate autophagy activity, which removes excess cellular ROS by activating transcription factors to degrade damaged organelles and proteins. Thus, autophagy can act as an antioxidant to protect cells from oxidative stress [18]. AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) is essential in regulating autophagy [21]. Recent studies have confirmed that SIRT1 regulates autophagy and AMPK. AMPK signaling response is cellular stress-sensitive to reduce oxidative stress and increase autophagy [22]. Increased mTOR signals diminish memory storage capacity [23], and cognitive-disease mouse models indicate escalated mTOR expression [24].

Various studies on neurodegenerative disease prevention and treatment using natural products have discovered safe and various dietary-derived candidates. For example, a recent


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study reported that phytochemicals such as limonoids, epigallocatechin-3-galate, and berberine alleviated neurodegenerative diseases [25]. Hesperetin is an aglycone hesperidin form and a representative flavonoid component primarily in citrus fruit and citron [26]. Hesperetin exhibits protective effects on the vascular system, anticancer, antibacterial, and antiallergic [27]; however, its neuroprotective effects in $H_2O_2$-induced neurodegenerative diseases remain unknown. Thus, we investigated hesperetin's protective effects and precise mechanisms against $H_2O_2$-induced oxidative damage in SH-SY5Y neuroblastoma cells.

## MATERIALS AND METHODS

### Materials

Hesperetin and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Aldrich (St. Louis, MO, USA). Quantitative polymerase chain reaction (qPCR) primers were procured from Bioneer (Daejeon, Korea). Thermo Fisher Scientific (Waltham, MA, USA) provided the bicinchoninic acid (BCA) protein assay kit. Unless otherwise stated, all other chemicals were purchased from Sigma Aldrich or Biosesang (Seongnam, Korea).

### Hesperetin and $H_2O_2$ treatment in human SH-SY5Y cells

Human SH-SY5Y cells were procured from the Korean Cell Line Bank (Seoul, Korea). Cells were cultured in Dulbecco's modified Eagle's medium-F12 supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin (Welgene, Daegu, Korea) and maintained at 37°C in a humidified 5% $CO_2$ atmosphere. Neuronal cells were seeded at a $1 \times 10^5$ cells/mL density. SH-SY5Y cells were pretreated with hesperetin 2 h before 24 h 400 μM $H_2O_2$ exposure.

### Measuring cell viability

The MTT assay measured hesperetin's cytotoxic effects on SH-SY5Y cells. First, cell viability was determined by adding the MTT solution (100 μL; 1 mg/mL) and a 2 h incubation. Next, the precipitated formazan was solubilized in 1 mg/mL of 100% dimethyl sulfoxide. Finally, plates were placed in a plate reader to measure absorbance at 570 nm.

### Measuring nitric oxide (NO) production

The Griess reaction measured the medium's NO production [28]. Each cell-free culture supernatant (100 μL) was mixed with an equal Griess reagent volume (1% sulfanilamide and 1% naphthyl ethylenediamine dihydrochloride in 30% acetic acid) for 15 min. An EZRead 400 Microplate reader (Biochrom, Cambridge, UK) determined the mixture's absorbance at 562 nm. The nitrite concentration was calculated using a sodium nitrite dilution as the standard curve.

### Measuring intracellular ROS levels

A DCFDA cellular ROS detection assay kit (Abcam, Cambridge, UK) measured intracellular ROS following the manufacturer's protocol. Cells were incubated with DCFDA for 45 min at 37°C. A microplate fluorescence reader (Tecan, Zurich, Switzerland) determined ROS distribution at Ex/Em = 485/535 nm.

### Immunoblotting analysis

A RIPA buffer (Biosesang) and the Halt™ protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific) prepared whole-cell lysates. Nuclear lysates were prepared using a nuclear extraction buffer (20 mM HEPES, 0.4 mM NaCl, 1 mM ethylenediaminetetraacetic


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acid, 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) containing 10% NP-40. A BCA protein assay (Pierce, IL, USA) is used to measure lysate protein concentrations following the manufacturer's protocol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis separated proteins (20 μg), and protein bands were transferred onto a nitrocellulose membrane (Invitrogen, Waltham, MA, USA), reacted for 2 h in a blocking buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20, and 5% nonfat dry milk), and incubated with appropriate primary antibodies for 2 h. After incubation, blots were washed thrice and incubated with diluted conjugated secondary antibody for 2 h. The Chemidoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA) applied a Western blotting luminol reagent (Santa Cruz Biotechnology, Dallas, TX, USA) to analyze results. The TNF-α, COX-2, phosphorylated (p)-IκBα, NF-κB, Bax, Bcl-2, p-p38 mitogen-activated protein kinase (p38), AMPK, mTOR, p-mTOR, and β-actin were purchased from Santa Cruz Biotechnology. Caspase-3, p-extracellular signal-regulated kinase (ERK), p-c-Jun N-terminal kinase (JNK), p-AMPK, and SIRT1 were purchased from Cell Signaling Technology (Beverly, MA, USA). Protein expression intensity was normalized to β-actin and quantified using ImageJ (a free online image analysis software).

## Real-time qPCR analysis

Following the manufacturer's protocol, total RNA was isolated from the cultured cells using a Trizol reagent (Thermo Fisher Scientific). Total RNA concentration and purity were assessed through 260 and 280 nm absorbance measurements using a NanoDrop 2000 (Thermo Fisher Scientific). An Omniscript RT kit (QIAGEN, Hilden, Germany) is used to synthesize first-strand cDNA from 1 μg of total RNA. SYBR green-based qPCR was performed using a real-time PCR detection system (Bio-Rad). The primers designed with an online program (Bioneer) were as follows: human *Bax*, forward 5′-TCCACCAAGAAGCTGAGCGAG-3′ and reverse 5′-GTCCAGCCCATGATGGTTCT-3′; *Bcl-2*, forward 5′-TCCGCGTGATTGAAGACACC-3′ and reverse 5′-TCTCCCGGTTATCGTACCCT-3′; *NF-κB*, forward 5′-GACAAGGTGCAGAAAGATGACAT-3′ and reverse 5′-TCATACGGTAACACAAGGCCT-3′; *β-actin* forward 5′-CACCCCGTGCTGCTGAC-3′ and reverse 5′-CCAGAGGCGTACAGGGATAG-3′. All reactions were run in triplicate. Significance was determined from β-actin-normalized $2^{-\Delta\Delta CT}$ value comparisons.

## Nuclear morphology assay

Cells were fixed in 4% paraformaldehyde (PFA) for 30 min and then washed with phosphate-buffered saline (PBS). Subsequently, cells were stained for 10 min in a freshly prepared 4′,6-diamidino-2-phenylindole (DAPI, 100 ng/mL; Beyotime, Shanghai, China) solution. Finally, the mounting solution was dropped onto a glass slide, a cover glass was placed over it, and nuclei were observed at 400× magnification via a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

## Immunofluorescence staining

After hesperetin treatment, cells were washed twice in PBS, fixed with 4% PFA for 30 min at 4°C, and stained overnight with the NF-κB antibody (1:100 dilution; Santa Cruz Biotechnology). Following air drying, slides were incubated with the secondary antibody (1:2,000 dilution; Invitrogen) for 60 min. Nuclei were stained with DAPI (100 ng/mL; Beyotime), and samples were washed thrice with PBS. The DAPI solution was incubated at 37°C for nucleic acid detection. Slides were washed twice in PBS, air-dried, treated with a mounting medium, and examined at 400× magnification under a fluorescence microscope. Leica Application Suite X software collected images.


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### Enzyme-linked immunosorbent assay (ELISA)

Cell-free supernatants were collected, and cytokine levels were measured using IL-6 and TNF-α ELISA kits (Raybiotech, Norcross, GA, USA) to determine hesperetin's effect on $H_2O_2$-treated SH-SY5Y cell cytokine production. Values were calculated based on a standard curve.

### Statistical analysis

All experiments were repeated at least three times, and each experiment's data were expressed as mean ± standard deviation. Significant differences among groups were determined by one-way analysis of variance, followed by the Duncan multiple range test with SPSS version 25.0 (IBM Corp., Armonk, NY, USA). Statistical significance was defined as $P < 0.05$. Specific significance values are stated in the figure legends.

## RESULTS

### Hydrogen peroxide's effect on human neuroblastoma SH-SY5Y cells

Cells were exposed to $H_2O_2$ at different concentrations to determine $H_2O_2$ cytotoxicity. Our results indicated that 400 μM $H_2O_2$ triggered approximately 50% of cell population death within 24 h (or the half maximal inhibitory concentration [$IC_{50}$] value). Therefore, we used 400 μM of $H_2O_2$ as the cell damage dose (**Fig. 1A**). The non-toxic hesperetin 10–40 μM concentration range was used in subsequent experiments. As shown in **Fig. 1B**, hesperetin increased cell viability in $H_2O_2$-treated SH-SY5Y cells compared to $H_2O_2$ alone. Thus, these non-toxic hesperetin concentrations and $H_2O_2$ $IC_{50}$ dosages were used for subsequent experiments.

### Hesperetin's effect on ROS and NO production levels in $H_2O_2$-treated SH-SY5Y cells

We investigated whether hesperetin suppressed ROS and NO production in $H_2O_2$-treated SH-SY5Y cells using DCFDA-DA and NO assays. As shown in **Fig. 2A**, intracellular ROS escalated in the $H_2O_2$-only treatment group compared to the untreated. Conversely, this increased ROS declined due to hesperetin treatment. In addition, $H_2O_2$ treatment intensified NO production, while hesperetin treatment abated NO production (**Fig. 2B**). Therefore, our results substantiate that hesperetin dose-dependently reduces intracellular ROS and NO production levels significantly ($P < 0.05$).

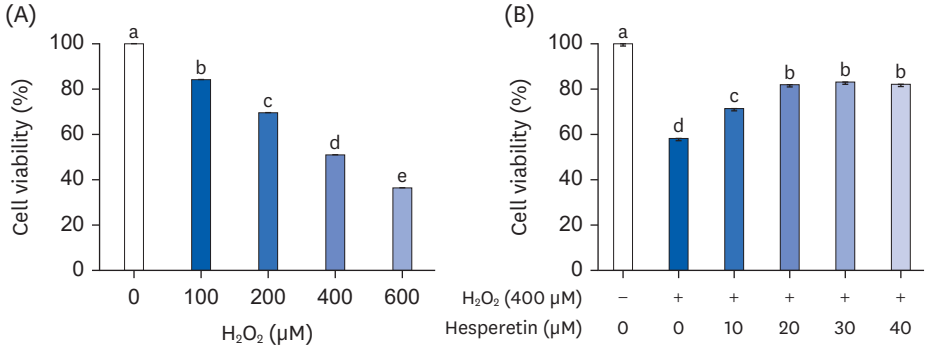


**Fig. 1.** Hesperetin increased cell viability in $H_2O_2$-treated SH-SY5Y cells.
(A) Cells were exposed to various $H_2O_2$ concentrations (100-600 μM) for 24 h, and the MTT assay was used to measure cell viability. (B) Cells were pretreated with hesperetin at several concentrations and then induced with or without 400 μM $H_2O_2$ for 24 h. Experiments were performed in triplicate, and results are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.
$H_2O_2$, hydrogen peroxide; MTT, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.


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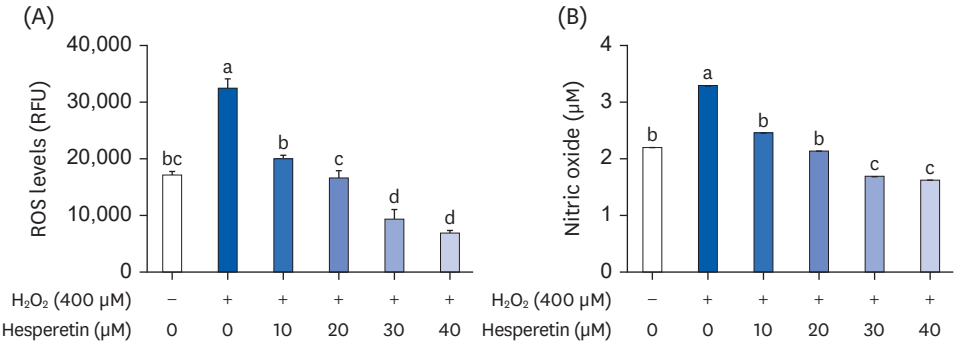


**Fig. 2.** Hesperetin suppressed ROS and NO production levels in $H_2O_2$-induced SH-SY5Y cells.
(A) The DCFDA assay detected intracellular ROS accumulation. A fluorescence plate reader estimated intracellular ROS levels at Ex/Em = 485/535 nm. (B) SH-SY5Y cells were treated with hesperetin (10–40 μM) for 2 h and then exposed to $H_2O_2$ for 24 h before harvest. The culture supernatant was assayed using the Griess reagent to estimate the NO concentration. Experiments were performed in triplicate, and results are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.
$H_2O_2$, hydrogen peroxide; ROS, reactive oxygen species; NO, nitric oxide.

## Hesperetin's effect on pro-inflammatory cytokine release and related gene expression via NF-κB pathway in $H_2O_2$-treated SH-SY5Y cells

We investigated inflammatory cytokines secretion and NF-κB expression from hesperetin treatment in $H_2O_2$-treated SH-SY5Y cells. ELISA assays revealed that inflammatory cytokines TNF-α and IL-6 secretions were significantly increased in $H_2O_2$-treated SH-SY5Y cells but were suppressed by hesperetin ($P < 0.05$) (**Fig. 3A and B**). In addition, COX-2 and TNF-α protein expressions increased in $H_2O_2$-treated SH-SY5Y cells (**Fig. 3C**); by contrast, hesperetin treatment reduced them.

IκBα and NF-κB gene expressions, involved in NF-κB-mediated neuropathological pathways, were markedly affected under oxidative stress conditions [29]. Hesperetin treatment significantly reduced NF-κB and p-IκBα levels ($P < 0.05$) and also reduced the mRNA's *NF-κB* gene expression levels compared to $H_2O_2$ control cells ($P < 0.05$) (**Fig. 4A and B**). Moreover, immunofluorescence analysis further confirmed 40 μM hesperetin's inhibition against $H_2O_2$-induced p65 nuclear translocation (**Fig. 4E**). These results indicate that hesperetin is a potential inhibitor against $H_2O_2$-induced neuronal inflammation.

## Hesperetin's effects on apoptosis signaling in $H_2O_2$-treated SH-SY5Y cells

We used immunoblotting and qPCR to assess caspase-3, Bax, and Bcl-2 expression levels and investigate whether hesperetin decreased cell death by inhibiting apoptosis signaling in $H_2O_2$-treated SH-SY5Y cells. As shown in **Fig. 5A**, hesperetin increased caspase-3 protein expression in $H_2O_2$-treated SH-SY5Y cells. Proapoptotic factor Bax expression was increased by $H_2O_2$ treatment compared to untreated cells; however, hesperetin application reduced its expression in $H_2O_2$-treated SH-SY5Y cells. In addition, anti-apoptotic protein Bcl-2 expression levels were increased by hesperetin exposure compared to the $H_2O_2$-treated group (**Fig. 5C**). The Bax/Bcl-2 ratio was significantly lower in the hesperetin treatment group compared to the $H_2O_2$-treated ($P < 0.05$) (**Fig. 5D**).

Moreover, *Bax* mRNA levels were increased in $H_2O_2$-treated cells compared to untreated cells, but *Bcl-2* mRNA levels were decreased. However, *Bax* was significantly decreased, and *Bcl-2* was considerably increased by hesperetin treatment ($P < 0.05$) (**Fig. 5E and F**). Additionally, DAPI staining confirmed hesperetin's effect on $H_2O_2$-treated SH-SY5Y nuclear


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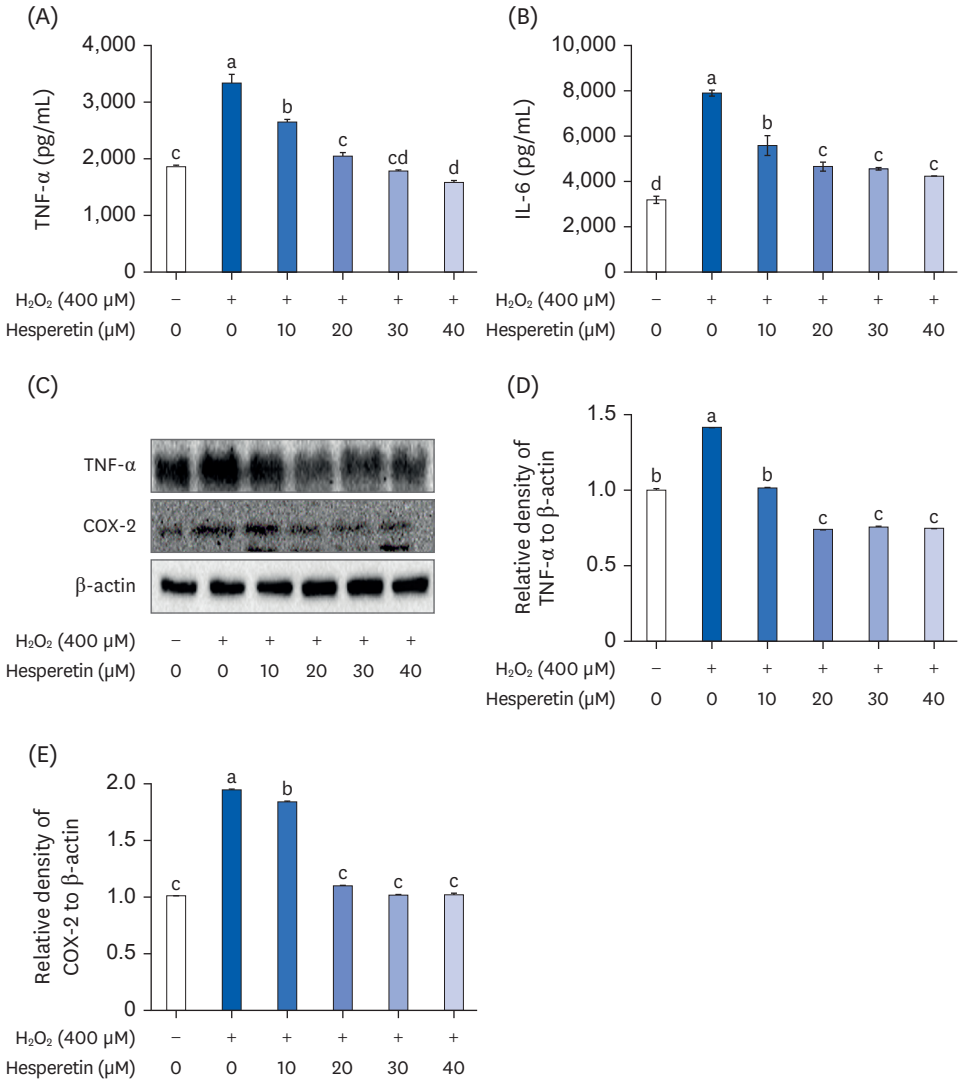


**Fig. 3.** Hesperetin inhibited inflammatory cytokine secretion factor in $H_2O_2$-treated SH-SY5Y cells.
SH-SY5Y cells were treated with hesperetin (10–40 μM) for 2 h and then exposed to $H_2O_2$ for 24 h before harvest. (A) An ELISA kit measured IL-6 and TNF-α secretion. TNF-α and COX-2 expression levels were measured using (A) immunoblotting, and (D–E) densities were normalized to β-actin using ImageJ software. (D) TNF-α and (E) COX-2 levels. Experiments were performed in triplicate, and results are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.
$H_2O_2$, hydrogen peroxide; TNF, tumor necrosis factor; IL, interleukin; COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbent assay.

cell morphology. As displayed in **Fig. 5G**, DAPI use revealed apoptotic bodies and nuclear condensation after $H_2O_2$ treatment. Alternatively, these were later inhibited by hesperetin in SH-SY5Y cells treated with 400 μM $H_2O_2$ ($P < 0.05$).

## Hesperetin's effect on MAPKs activation in $H_2O_2$-treated SH-SY5Y cells

MAPK activation induces neuronal cell death [30]; thus, we observed how hesperetin affected it in $H_2O_2$-treated SH-SY5Y cells. As shown in **Fig. 6**, $H_2O_2$ increased ERK, JNK, and p38 phosphorylation, whereas hesperetin suppressed them. These results indicated that hesperetin suppressed cell death via p-ERK, p-JNK, and p-p38 activation.


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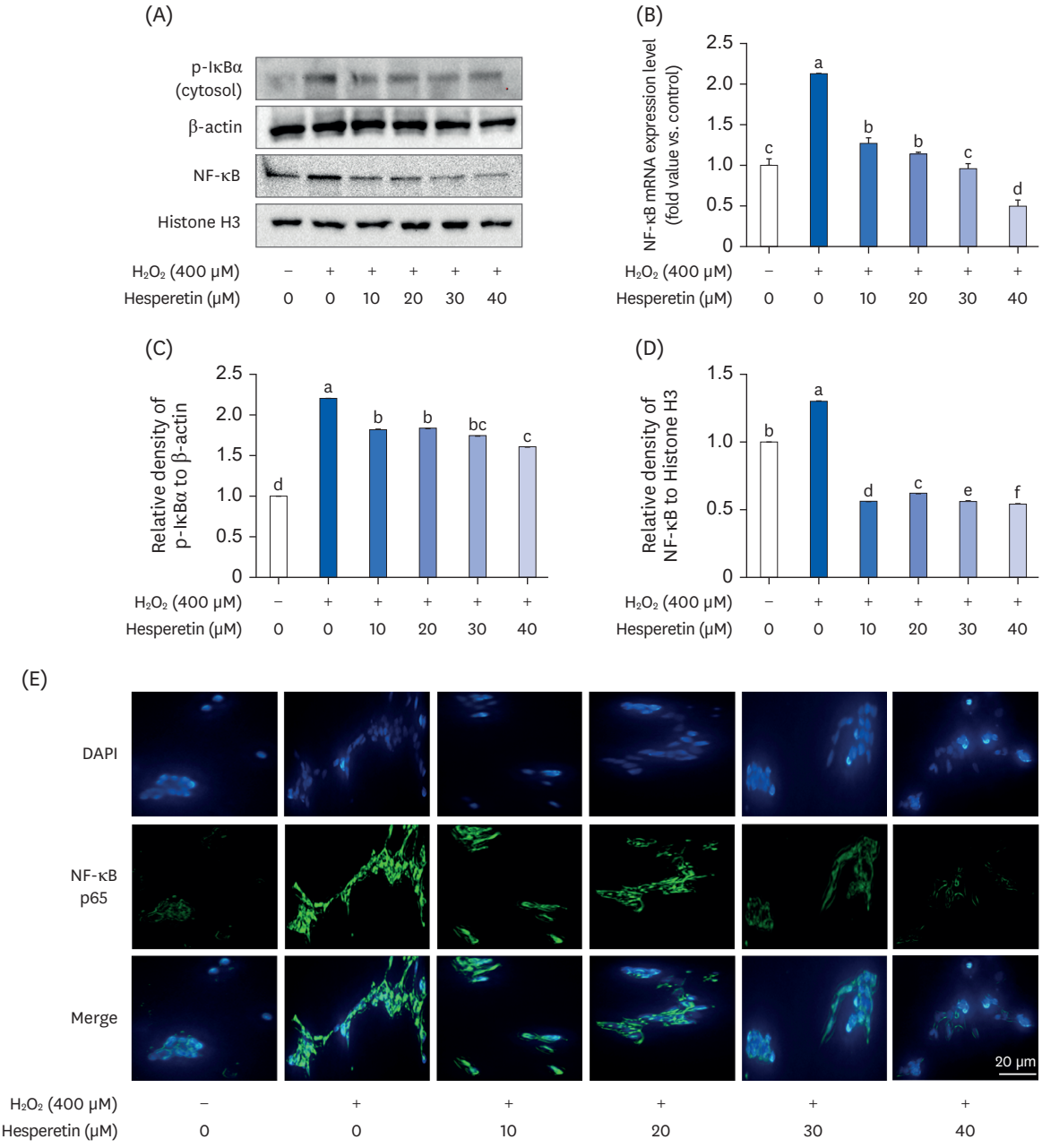


**Fig. 4.** Hesperetin's effects on NF-κB activation in $H_2O_2$-treated SH-SY5Y cells.
NF-κB and p-IκBα proteins levels were measured using (A) immunoblotting, and (B-E) densities were normalized to β-actin using ImageJ software. (B) p-IκBα (cytosol), (C) NF-κB (nuclear). Cells were harvested, and (D) NF-κB mRNA expression in $H_2O_2$-induced SH-SY5Y cells was evaluated. Data are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test. (E) SH-SY5Y cells were treated with hesperetin and fixed with 4% paraformaldehyde. After blocking with an appropriate buffer, cells were incubated with antibodies. Next, DAPI staining confirmed cell nuclei. Signals were quantified using fluorescence microscopy at 400× magnification.
$H_2O_2$, hydrogen peroxide; NF, nuclear factor; p-, phosphorylated; DAPI, 4′,6-diamidino-2-phenylindole.


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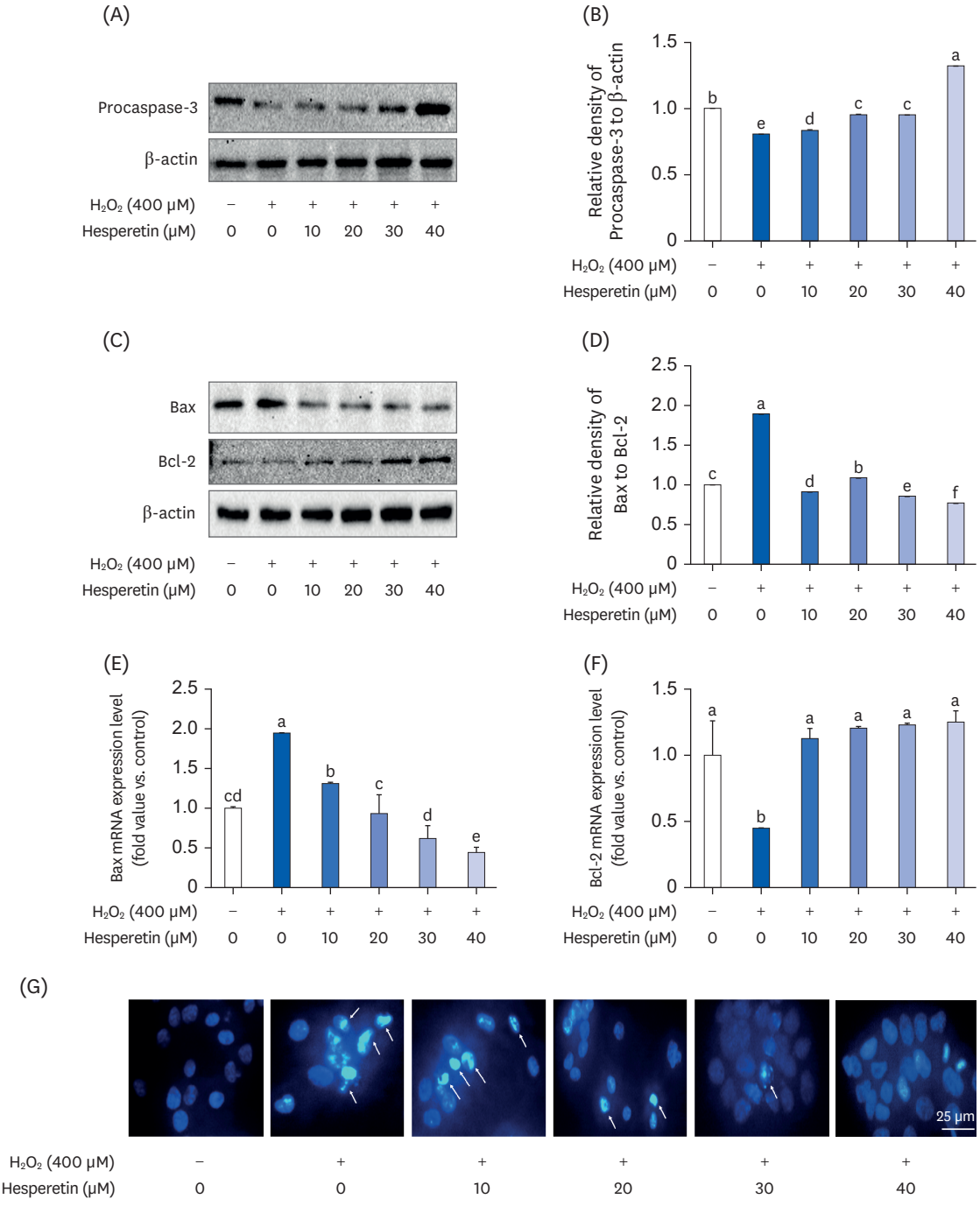


**Fig. 5.** Hesperetin inhibited $H_2O_2$-induced apoptosis in $H_2O_2$-treated SH-SY5Y cells.
Procaspase-3 protein expression levels were determined using (A) immunoblotting, and (B) densities were normalized to β-actin using ImageJ software. Bax and Bcl-2 protein expression levels were measured using (C) immunoblotting, and (D) densities were normalized to β-actin using ImageJ software. (D) Bax/Bcl-2 levels. (E, F) The relative mRNA expression levels are depicted after normalization against β-actin mRNA expression. The data are expressed relative to untreated cells' mRNA levels, which was arbitrarily defined as 1. Experiments were performed in triplicate, and the results are presented as the mean ± SD. Data were analyzed by applying the $2^{-\Delta\Delta CT}$ method. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test. (G) SH-SY5Y cells were treated with hesperetin (10–40 μM) for 24 h and fixed with 4% paraformaldehyde. A fluorescence microscope assessed signal quantification at 400× magnification.
$H_2O_2$, hydrogen peroxide; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.


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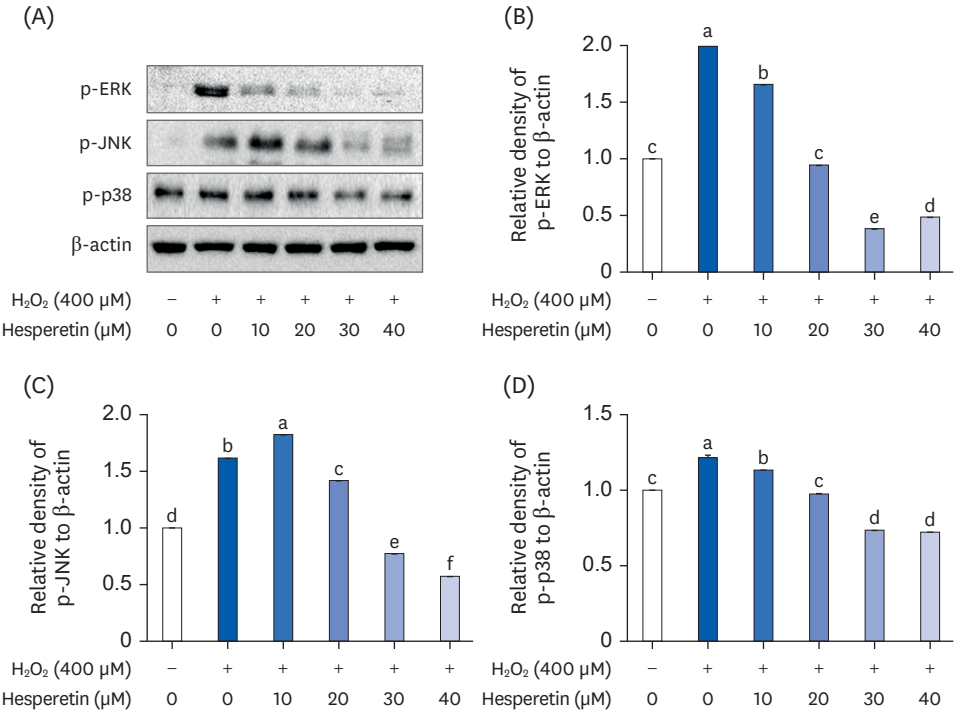


**Fig. 6.** Hesperetin's effect on the MAPK signaling pathway in $H_2O_2$-treated SH-SY5Y cells.
p-ERK, p-JNK, and p-p38 expression levels were measured using (A) immunoblotting, and (B-D) densities were normalized to β-actin using ImageJ software. (B) p-ERK, (C) p-JNK, and (D) p-p38 levels. Experiments were performed in triplicate, and results are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.
MAPK, mitogen-activated protein kinase; $H_2O_2$, hydrogen peroxide; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; p38, p38 mitogen-activated protein kinases.

### Hesperetin's effect on autophagy-modulating protein expression in $H_2O_2$-treated SH-SY5Y cells

Next, we examined hesperetin's effect on autophagy in $H_2O_2$-treated SH-SY5Y cells. AMPK-mTOR is a representative autophagy signaling pathway. As shown in **Fig. 7**, $H_2O_2$ downregulated AMPK phosphorylation and SIRT1 expression and upregulated mTOR activation, whereas hesperetin alleviated these autophagy-modulating protein expressions. In summary, hesperetin downregulated mTOR activation and upregulated SIRT1 and AMPK phosphorylation.

## DISCUSSION

Progressive cognitive function loss and memory, reasoning, and language decline characterize neurodegenerative diseases [31]. In addition, oxidative stress also characterizes acute and chronic illnesses, including neurodegenerative diseases [32]. Increased neuronal ROS and oxidative stressors exacerbate blood-brain barrier (BBB) permeability, leading to synaptic function loss and cell death [33,34]. Natural dietary agent studies focus on neuroprotection and regeneration, such as curcumin, silibinin, and chlorogenic acids [35-38]. For instance, hesperetin is a dietary compound and an aglycone-hesperidin citrus polyphenol flavonoid used in cardiovascular disease and cancer treatments [39].


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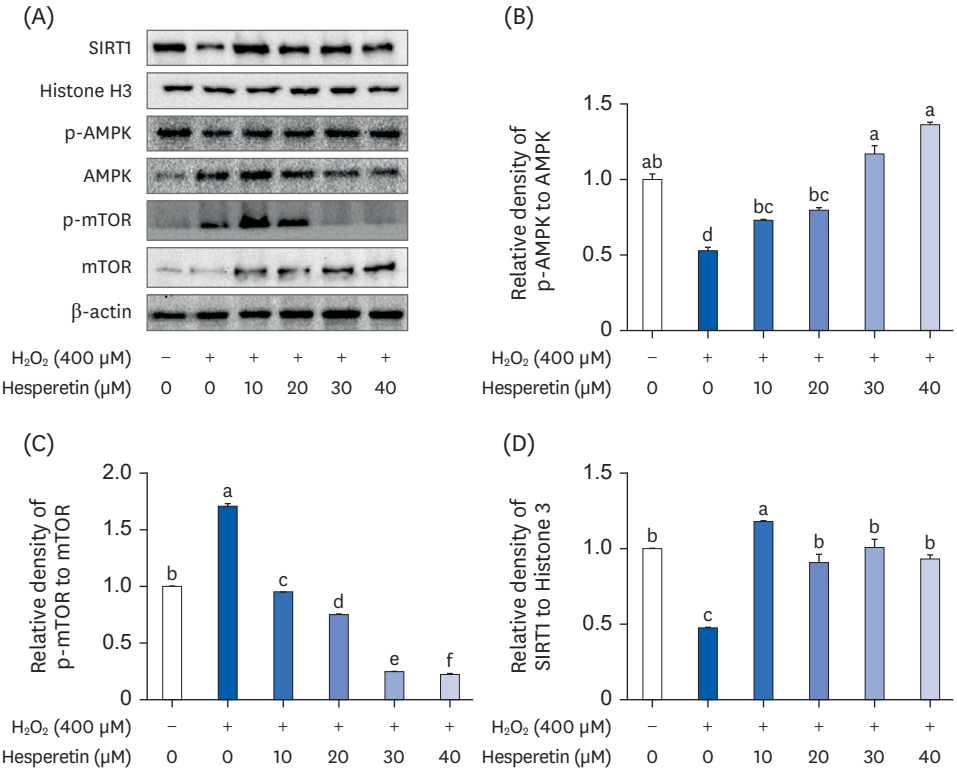


**Fig. 7.** Hesperetin induces autophagy through the SIRT1-AMPK-mTOR pathway.
SIRT1-AMPK-mTOR pathway protein expression levels were measured using (A) immunoblotting, and (B-D) densities were normalized to β-actin using ImageJ software. (B) p-AMPK/AMPK, (C) p-mTOR/mTOR, and (D) SIRT1 (nuclear) levels. Experiments were performed in triplicate, and results are presented as the mean ± SD. Different letters indicate significant differences ($P < 0.05$) as determined by Duncan's multiple range test.
$H_2O_2$, hydrogen peroxide; AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; Sirt1, NAD-dependent deacetylase sirtuin-1; p-, phosphorylated.

$H_2O_2$ is a metabolite and oxidative stress that passes through biological membranes and causes cell damage [40]. Furthermore, $H_2O_2$ accumulation, brain inflammation, and apoptosis are observed in neurodegenerative diseases [41]. We confirmed that the cell viability was decreased dose-dependently in $H_2O_2$-treated SH-SY5Y cells. However, SH-SY5Y cell viability with $H_2O_2$ incubation was significantly increased when pretreated with hesperetin, demonstrating hesperetin's neuroprotective effect on $H_2O_2$-treated SH-SY5Y cells. Ma *et al.* [42] reported that proanthocyanidins, flavonoids derived from plants, attenuated rotenone-induced ROS production in SH-SY5Y cells. Ling *et al.* [43] reported that asiaticoside suppressed ROS and NO in $H_2O_2$-treated SH-SY5Y cells. Our study corroborates other findings that ROS and NO production in $H_2O_2$-treated SHSY5Y cells was increased and was significantly reduced by hesperetin treatment.

Abnormal protein accumulation modified by nerve cell damage triggers inflammatory responses and secretes inflammatory mediators to cause neurotoxicity and brain tissue destruction, exacerbating neurodegenerative diseases [7,44]. iNOS and COX-2 are expressed, and PGE2 and NO are excessively produced in ROS-induced inflammation [12]. These inflammatory initiation factors (iNOS, IL-6, TNF-α) cause neuro damage when activated NF-κB promotes transcription and excessive NO production [45]. NF-κB is a representative inflammatory cell signaling factor regulating inflammatory gene substances such as cytokines and chemokines [46]. NF-κB binds to IκB and inactively resides in the cytoplasm.


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As ROS and external stimuli separate it from IκB, subunit p65 moves to the nucleus to increase COX-2, IL-6, and TNF-α [47,48].

Our study confirmed that $H_2O_2$ upregulated TNF-α, COX-2, and NF-κB protein expressions in SH-SY5Y cells, indicating that $H_2O_2$ could activate inflammatory responses. Conversely, hesperetin treatment downregulated NF-κB, COX-2, and TNF-α expression. Park *et al.* [49] reported that *Petalonia binghamiae* extract regulated inflammatory COX-2, p-IκB, and NF-κB factors to inhibit inflammatory responses in LPS-induced microglia BV2 cells. In a recent study, inflammatory cytokines in cellular and animal models exhibit inhibition from natural products such as luteolin, quercetin, and resveratrol [50]. These findings propose that hesperetin suppresses $H_2O_2$-induced neuroinflammation by regulating NF-κB expression.

Oxidative stress-induced neuronal cell death is related to the mitochondria-related apoptosis pathway and is the most prominent neurodegenerative diseases [1]. Additionally, the Bax/Bcl-2 ratio is crucial in apoptosis. Recent studies reported that flavonoids, such as bailarein, kaempferol, quercetin, and naringenin, modulated the mitochondrial apoptosis pathway, activated signal pathways to suppress apoptogenic Bax, and induced antiapoptotic proteins [51]. Park *et al.* [52] determined that *Glycyrrhiza uralensis* Radix extract inhibited apoptosis through caspase-3, Bax, and Bcl-2 regulation in $H_2O_2$-induced glial C6 cells. Similarly, we confirmed that hesperetin upregulated Bcl-2 and caspase-3 and downregulates Bax in $H_2O_2$-treated SH-SY5Y cells. In addition, hesperetin treatment inhibited Bax/Bcl-2 ratio escalation.

Neurodegenerative disorder apoptosis is characterized by morphological changes such as cell shrinkage, chromatin condensation, and nucleosome degradation [53]. DAPI staining validated that hesperetin slightly attenuated apoptotic feature induction compared with $H_2O_2$ treatment alone. Oxidative stress activates the MAPK pathway, incorporating ERK, JNK, and p38 [54]. ROS is related to MAPK pathway activation, especially in neurons; oxidative stress activates JNK and p38 signaling pathways [55,56]. In addition, ERK is essential for enhancing neuronal cell death [57,58].

Excessive ROS activates MAPKs in the brain, inducing neuronal cell death or neurodegenerative diseases [59,60]. MAPKs are signaling systems that mediate biological responses such as cell proliferation, death, and differentiation when cell membrane receptors are activated by extracellular stimuli [61]. MAPK signaling activation increases the Bax/Bcl-2 ratio and curtails mitochondrial membrane potential, promoting cytochrome C release and caspase activation [62]. Lee *et al.* [63] reported that red ginseng oil attenuated the MPAK signaling pathway in Aβ25-35-treated PC12 cells. Tang *et al.* [64] elucidated that sabinin could reduce ERK, JNK, and p38 activities and inhibit neuroinflammation in the LPS-induced mouse hippocampus. Similarly, our results verified that hesperetin pretreatment suppressed ERK, JNK, and p38 in $H_2O_2$-treated SH-SY5Y. Several studies have established that autophagy has a significant neuroprotective role in neurodegenerative diseases [65]. Kim *et al.* [66] reported that chebulagic acid enhances autophagy through AMPK/mTOR/Beclin-1 regulation in MPP-induced SH-SY5Y cells. Additionally, Tao *et al.* [67] concluded that phloretin improved Parkinson's disease symptoms through autophagy pathway regulation in a rotenone-induced Parkinson's disease mouse model.

Furthermore, autophagy is prominent in AMPK/mTOR pathway regulation, and the AMPK/mTOR signaling pathway is necessary for autophagy and apoptosis [68]. When phosphorylation increases, AMPK decreases mTOR (a downstream AMPK target) levels


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which is essential for apoptosis and autophagy. AMPK activates autophagy and plays a crucial role in synaptic plasticity and memory formation by controlling the mitochondrial metabolic rate in neuronal synaptic activation, thereby maintaining neuronal energy at a certain level [69]. Autophagy inhibitor mTOR also affects learning and memory by modulating synaptic plasticity, and mTOR signaling dysregulation leads to neuronal dysfunction, apoptosis, and decreased memory storage capacity [70,71]. Autophagy activation through mTOR regulation exhibits neuroprotective effects in various neurological diseases [72]. Additionally, AMPK regulates complex mitochondrial biosynthesis signal transduction through SIRT1 and mTOR [73]. SIRT1 regulates autophagy by participating in autophagosome formation and regulating chronic disease pathogenesis, such as neurodegenerative and cardiovascular diseases. Also, Wang *et al.* [74] reported that SIRT1/AMPK interaction was integral for Aβ accumulation and cognitive functions in Alzheimer's disease.

Recent *in vitro* and *in vivo* studies report that natural compounds such as quercetin, conophylline, and limonene regulate autophagy and inhibit neurodegenerative diseases [75]. For example, Wu *et al.* [76] reported that resveratrol modulates the SIRT1/AMPK/mTOR pathway to induce mitochondrial autophagy, protecting Parkinson's disease. Furthermore, Chen *et al.* [77] discovered that quercetin protects neurons by activating the AMPK pathway against okadaic acid-induced neurotoxicity in SH-SY5Y cells. In addition, Zhao *et al.* [78] determined that glutamine inhibited the AKT/mTOR pathway to relieve oxidative stress from MPP-induced neurotoxicity in PC12 cells. Moreover, epigallocatechin-3-gallate and resveratrol improved learning and spatial memory by activating AMPK in animal cognitive impairment [79,80].

Post-mortem brain examinations of Alzheimer's patients revealed increased mTOR signaling [81,82]. It has also been suggested that over-activated mTOR signaling in the hippocampus and neurons of aged mice contributes to neurodegenerative disorders [83]. Gao *et al.* [84] reported that chlorogenic acid reduced mTOR signaling in cognitive dysfunction and ameliorated impairment in. In our study of $H_2O_2$-treated SH-SY5Y cells, SIRT1 and p-AMPK levels were decreased, whereas p-mTOR expression was increased. However, hesperetin pretreatment activated SIRT1 and p-AMPK and suppressed p-mTOR.

These results confirm that hesperetin suppresses apoptosis by inhibiting the MAPK signaling pathway. Furthermore, hesperetin treatment in $H_2O_2$-treated SH-SY5Y cells inhibits inflammation-related (TNF-α, IL-6) and NF-κB target gene expressions. Hesperetin also activates autophagy through AMPK/mTOR, an autophagy regulator. Therefore, hesperetin can protect against $H_2O_2$-induced oxidative damage in SH-SY5Y cells. Our results substantiate that hesperetin is a promising natural candidate for protecting neurons from oxidative stress and preventing neurodegenerative diseases.

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