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EXPERIMENTAL STUDY

# Epigallocatechin-3-gallate and resveratrol attenuate hydrogen peroxide induced damage in neuronal cells

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**ABSTRACT**

PURPOSE: The purpose of this study is to investigate how the antioxidants resveratrol and epigallocatechin-3-gallate (EGCG) protect SH-SY5Y cells against damage caused by hydrogen peroxide ($H_2O_2$).

METHODS: SH-SY5Y cells were pretreated with EGCG and resveratrol at concentrations of 0.1 μM**,** 1 μM, and 10 μM, individually and in various combinations. Cells were exposed to 250 μM $H_2O_2$ for 1-hour following a 24-hour pretreatment. The effects of EGCG and resveratrol on cellular survival against hydrogen peroxide toxicity were evaluated with the MTS. Caspase 3 levels were measured with caspase 3 ELISA test for evaluating survival. The clonogenic test was utilized to assess the colony forming capacity.

RESULTS: The MTS test revealed that pretreatment of SH-SY5Y cells for 24 hours with EGCG and Resveratrol enhanced cellular survival against hydrogen peroxide damage at all dosages ($p < 0.005$)**.** The caspase 3 ELISA test revealed that EGCG and resveratrol significantly decreased caspase 3 levels and improved cellular survival via the caspase 3 pathway ($p < 0.005$). The clonogenic test findings show that resveratrol and EGCG statistically boost SH-SY5Y cells' potential to form colonies ($p<0.005$)**.**

CONCLUSIONS: By reducing caspase 3 levels in exposure to hydrogen peroxide damage, EGCG and resveratrol promote cellular viability *(Tab. 1, Fig. 3, Ref. 37)*. Text in PDF *www.elis.sk*

KEY WORDS: epigallocatechin-3-gallate, resveratrol, hydrogen peroxide, neurodegeneration.

## Introduction

When neurons in a specific area of the brain are damaged or lost, neurodegenerative disorders, which are progressive and irreversible, result (1). Major illnesses brought on by neurodegeneration include Alzheimer's and Parkinson's disease. Recent research has focused on the molecular processes that might contribute to the development of neurodegenerative disorders. In the mammalian brain, neurons are strongly dependent on oxidative metabolism for ATP production, and therefore, are prone to ROS-dependent damage. The accumulation of reactive oxygen species (ROS) including superoxide, singlet oxygen, hydroxyl radical and hydrogen peroxide ($H_2O_2$) leads to oxidative stress (2) . In terms of the development of neurodegenerative diseases, oxidative stress and neuroinflammation are among the most significant variables (3). An imbalance between ROS production and antioxidants occurs in Alzheimer's disease (AD), Parkinson's disease (PD), and other types of dementia of the elderly (4).

Flavonoids, which are included in the scope of nutraceutical antioxidants, are polyphenolic secondary metabolite phytochemicals obtained from natural food sources and have certain therapeutic value and open the door to the use of alternative strategies to overcome neurodegenerative diseases such as PD and AD. Epigallocathechin gallate (EGCG) and resveratrol are two of the most frequently studied dietary flavonoids. Green tea has been widely consumed in Asian countries, and an inverse relation between tea consumption and the incidence of dementia, PD, or Alzheimer disease has been reported (5, 6). The most prevalent (at least 50 %) antioxidant in green tea is epigallocatechin-3-gallate (EGCG), a naturally occurring polyphenolic catechin (7–9). Most of the studies have been conducted administrating green tea extracts or pure EGCG (8). Studies have revealed that EGCG possesses anticancer, anti-diabetes, anti-inflammatory, anti-cardiovascular, and anti-neurodegenerative properties (10–12). Fruits including grapes, strawberries, and peanuts contain resveratrol, another antioxidant (13). Resveratrol has been shown in studies to have anti-inflammatory, anticarcinogenic, and neuroprotective properties (14, 15).

Human neuroblastoma cells called SH-SY5Y are utilized in neurotoxicity studies as a dopaminergic neuronal cell line (16). Several studies have demonstrated the neuroprotective effects of Epigallocatechin gallate and resveratrol on neurodegeneration in both cell culture and animal models (15, 17–21). However, in SH-

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SY5Y cells, a derivate of the human SK-N-SH cells, the effects of two antioxidant compounds, resveratrol and epigallocatechin-3-gallate, against hydrogen peroxide damage has not been studied.

In this study, we tested the effects of EGCG and resveratrol against $H_2O_2$ damage on a homogeneous culture of neuroblastoma-derived SH-SY5Y cells. We sought herein to determine whether these organic substances have a protective effect against neuronal damage and how much they contribute to the apoptotic process.

## Materials and methods

*Chemicals*

SH-SY5Y cells were obtained from Sigma-Aldrich. Resveratrol and epigallokateşin-3-gallate, were purchased from Sigma-Aldrich. DMEM, Fetal Bovine Serum and Trypsin-EDTA (0.25 %) phenol red, were purchased from Gibco. $H_2O_2$ (Hydrogen peroxide solution) was obtained from Merck. The Caspase 3 ELISA kit was purchased from Sigma-Aldrich. MTS test was purchased from Promega.

*Cell culture*

As a dopaminergic cell line, the SH-SY5Y cell line was cultured in DMEM/F-12 (Dulbecco Modified Eagle Medium/Nutrient Mixture F-12) with 1 % Penicillin-Streptomycin and 10 % fetal bovine serum (FBS). Cultures were kept at 37 °C in a humidified atmosphere incubator with 5 % $CO_2$. When cell confluency reached 80 %, cells were harvested with 2 mL Trypsin-EDTA solution. Cells were seeded in 96-well plates at a density of 5x $10^4$ cells/00 µL per well for 24 h. The cells pretreated with different concentration of EGCG or/and resveratrol (0.1 µM, 1 µM, 10 µM) after 24 h incubation. Then the cells were treated with 250 µM $H_2O_2$ for 1 h.

*Cell viability analysis*

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) assay. After SH-SY5Y cells were exposed to $H_2O_2$, the cells were washed with phosphate-buffered saline (PBS) and incubated for 48 h with fresh culture medium. For MTS assay, MTS solution was prepared with MTS powder to PBS (20:1). After 48 h incubation, MTS solution was added 20 µL/well for 1 h. Subsequently, the optical density was evaluated at 490 nm using a Microplate Reader (ELISA reader-BIOTEK).

*Clonogenic assay*

SH-SY5Y cells were grown in 6-well plates at a density of 5x $10^4$ cells/well containing 2 mL of cell culture medium and incubated for 24 h. Subsequently, the cells were treated with resveratrol or epigallocatechin-3-gallate (0.1 µM, 1 µM, and 10 µM) for 24 h. In addition to single Epigallocatechin-3-gallate and resveratrol doses, the cells were treated with a mix concentration of 0.1 µM EGCG- 0.1 µM resveratrol, 1 µM EGCG- 1 µM resveratrol, 10 µM EGCG- 10 µM resveratrol for 24 h. After 24 h incubation, SH-SY5Y cells were treated with 250 µM $H_2O_2$ for 1 h. Subsequently, the cells were rinsed with PBS and incubated 7 days with fresh culture medium to examine their ability to produce colonies. After 7 days, culture medium was removed, and cells were washed with PBS. Subsequently, the cells were stained with crystal violet dye and the colony formation status of the cells was evaluated.

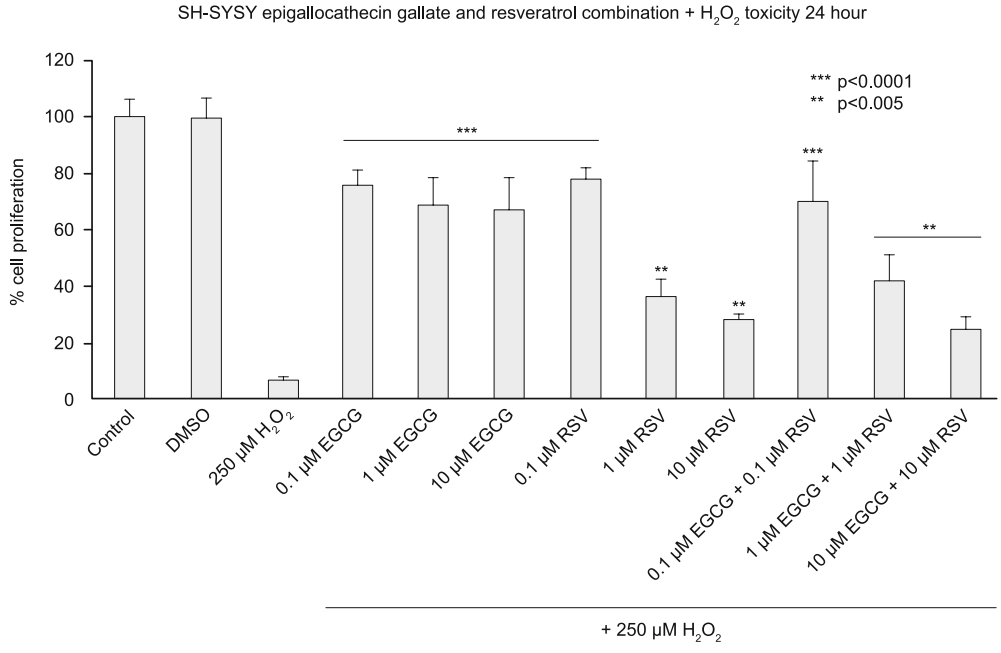


**Fig. 1. The effect of 24-hour pre-treatment with EGCG and Resveratrol on cell proliferation against hydrogen peroxide toxicity in SH-SY5Y cells.**

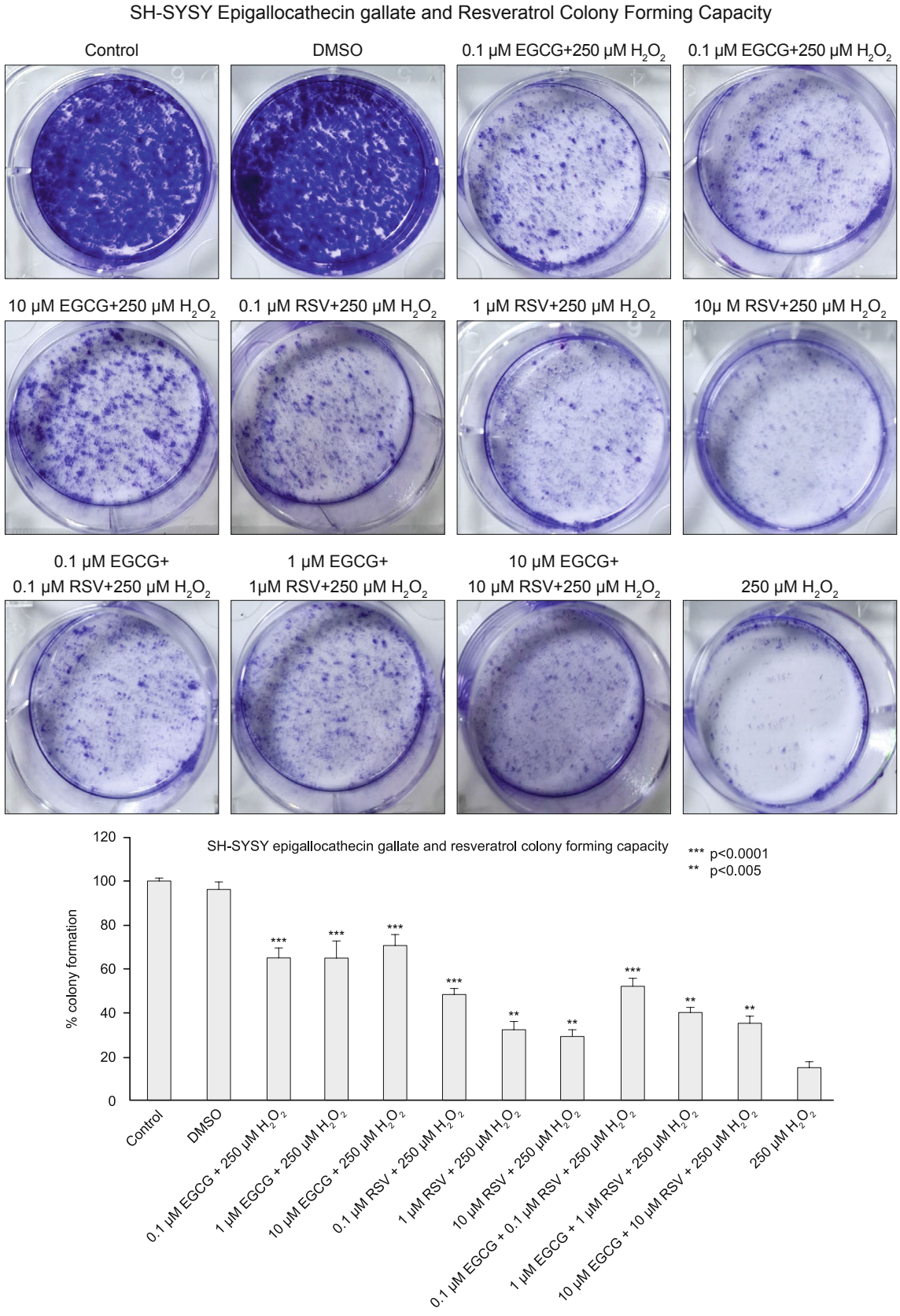


**Fig. 2. Effect of EGCG and resveratrol on colony forming capacity in SH-SY5Y cells against hydrogen peroxide toxicity.**

*Measurement of caspase-3 activity*

Caspase 3 activity was measured according to instructions of the caspase 3 ELISA assay (Sigma-Aldrich). Optical density was measured at 450 nm and the amount of caspase 3 calculated from a standard curve.

*Statistical analysis*

All data analysis was performed using SPSS 17.0. Comparisons between groups were performed by analyses of variance (ANOVA) test and followed by HSD post hoc comparison test. A p value of less than 0.05 ($p < 0.05$) was considered significant difference.

**Results**

In our study, we supposed that EGCG and resveratrol could improve the oxidative injury in SH-SY5Y cells induced by $H_2O_2$ by inactivating Caspase 3 pathways. To confirm the protective effects of EGCG and resveratrol against oxidative damage in $H_2O_2$-treated cells, we explored its effects on cell proliferation, reproduction/colony formation ability and apoptosis in $H_2O_2$-exposed cells.

*Cell viability*

Epigallocatechin-3-gallate and resveratrol pre-treatment for 24 hours protects SH-SY5Y cells against $H_2O_2$ toxicity

The outcomes of the MTS evaluation showed that all dosages of EGCG and resveratrol protected SH-SY5Y cells from hydrogen peroxide damage following the EGCG and resveratrol pretreatment treated SH-SY5Y cells at concentrations of 0.1 μM, 1 μM, and 10 μM ($p < 0.005$). Resveratrol was shown to have a greater protective impact on cell proliferation at a dosage of 0.1 μM ($p < 0.0001$) compared to other doses. The protective efficacy of the combination of EGCG and resveratrol against $H_2O_2$, an oxidative agent, in SH-SY5Y cells was statistically significant ($p < 0.005$). The combination of 0.1 μM EGCG and 0.1 μM resveratrol was discovered to be more protective than the other combined dosages of EGCG and resveratrol. ($p < 0.0001$) (Fig. 1).

*Reproduction/colony formation ability*

Resveratrol and EGCG boost SH-SY5Y cells' ability to form colonies in the presence of hydrogen peroxide toxicity

When SH-SY5Y cells were pretreated with EGCG and resveratrol at doses of 0.1 μM, 1 μM, and 10 μM against toxicity from 250 μM $H_2O_2$, it was revealed that their capacity to form colonies was statistically considerably higher than that of the toxicity group ($p < 0.005$). All dosages of EGCG and resveratrol statistically boosted the colony forming capability ($p < 0.005$), however the low dose combination of EGCG and resveratrol raised the colony percentage more than the other dose combinations ($p < 0.0001$) (Fig. 2). It was shown that among the groups in which resveratrol was provided alone, the low resveratrol dose (0.1 M) raised the colony percentage more than other resveratrol doses ($p < 0.0001$) (Fig. 2).

*Caspase-3 activity*

EGCG and resveratrol demonstrate protective activity against apoptosis by reducing caspase 3 levels against hydrogen peroxide damage in SH-SY5Y cells

Exposing the cells to $H_2O_2$ induced the expression of caspase-3. In comparison to the group that was just exposed to $H_2O_2$, all dosages of EGCG and resveratrol statistically substantially decreased

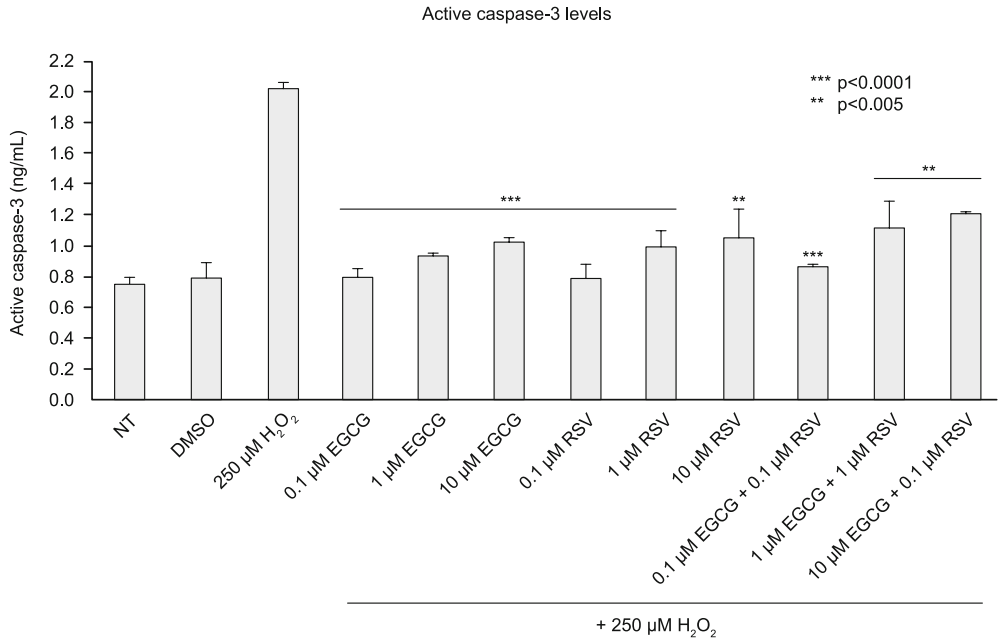


**Fig. 3. Effect of EGCG and resveratrol pre-treatment on caspase 3 protein against hydrogen peroxide toxicity in SH-SY5Y cells.**

**Tab. 1. Protein density in all groups as a result of apoptosis.**

| | Average | Standard deviation |
|---|---|---|
| NT | 0.754 | 0.049345 |
| DMSO | 0.792 | 0.103623 |
| 250μM H2O2 | 2.020 | 0.034541 |
| 0,1 μM EGCG | 0.803 | 0.049345 |
| 1 μM EGCG | 0.942 | 0 |
| 10 μM EGCG | 1.022 | 0.024672 |
| 0,1 μM Resveratrol | 0.803 | 0.078951 |
| 1 μM Resveratrol | 0.998 | 0.108558 |
| 10 μM Resveratrol | 1.050 | 0.182575 |
| 0,1 μM EGCG + 0,1 μM Resveratrol | 0.869 | 0.014803 |
| 1 μM EGCG + 1 μM Resveratrol | 1.117 | 0.167771 |
| 10 μM EGCG + 0,1 μM Resveratrol | 1.211 | 0.014803 |

caspase 3 levels in SH-SY5Y cells ($p < 0.005$). The 0.1 μM and 1 μM concentrations of resveratrol decreased caspase 3 levels more than the 10 μM concentration ($p < 0.0001$) (Fig. 3). Low dosage (0.1 μM) EGCG and resveratrol pre-treatment was observed to lower caspase 3 levels ($p < 0.0001$) more than other combination doses when the combination doses of both antioxidant substances were tested (Tab. 1).

**Discussion**

Epigallocatechin gallate and resveratrol are potent antioxidant molecules, and the literature contains abundant research exploring their benefits against various diseases (22, 23). Due to their antioxidant potency, these two compounds have been intensively investigated as anti-carcinogenic compounds; in addition, their neuroprotective properties are also the ongoing subject of research (24, 25). In the present study, we evaluated the capacity of EGCG and resveratrol to prevent $H_2O_2$-induced toxicity in SH-SY5Y cells. We explored the neuroprotective effects of EGCG and resveratrol on SH-SY5Y cells exposed to $H_2O_2$ by measuring the caspase 3 levels, which mark apoptosis. Our study supports others showing that resveratrol pretreatment can protect SH-SY5Y cells from the toxicity induced by H2O2 (26, 27). However, our study demonstrates for the first time to our knowledge that EGCG pretreatment can protect SH-SY5Y cells from toxicity induced by $H_2O_2$.

This research used a dopaminergic neuronal cell line of human neuroblastoma cells known as SH-SY5Y. We selected $H_2O_2$ to induce SH-SY5Y cell death. Hydrogen peroxide ($H_2O_2$) is one of the free oxygen species (ROS) and can cause neurodegeneration, as has been previously reported (28, 29). Exogenous $H_2O_2$ can enter cells and cause high oxidative stress due to high membrane permeability and is widely used by many investigators as a model of oxidative stress in SH-SY5Y cells (30, 31).

We observed that EGCG protected SH-SY5Y cells from hydrogen peroxide damage at concentrations of 0.1 μM, 1 μM, and 10 μM following pretreatment; we observed resveratrol to protect SH-SY5Y cells at the same doses but found resveratrol to exert more protective effect on cell viability at 0.1 μM compared to other doses. In addition, we found the combination of 0.1 μM EGCG and 0.1 μM resveratrol to be more protective than other combined doses of EGCG and resveratrol (Figure 1). Consistent with these results, Resveratrol and EGCG enhanced the colony forming ability of SH-SY5Y cells in the presence of hydrogen peroxide-induced toxicity (Fig. 2).

Cao et al pre-treated pancreatic alpha (aTC1-6) cells with EGCG at doses of 10 μM, 30 μM, 100 μM, and 300 μMa and looked at cell survival in the subsequent presence of hydrogen peroxide toxicity. The researchers revealed that EGCG protects pancreatic alpha cells from hydrogen peroxide damage, decreasing apoptosis and increasing cell survival (32). The EGCG concentrations used in our study to Protect SH-SY5Y cells against hydrogen peroxide damage were comparable to Ting Cao's work. We reached the conclusion that EGCG and resveratrol demonstrated the same effect by lowering intracellular caspase 3 levels as the result of a Caspase-3-ELISA test performed on EGCG and resveratrol. Jiao Meng et al examined the effects of EGCG against hydrogen peroxide toxicity in HUVECs cells and discovered that pre-treatment with EGCG occurs via the mTOR pathway and has a protective effect against $H_2O_2$ toxicity in HUVECs cells (33). While the results of our research and those of Jiao Meng et al are comparable, more thorough studies are needed to determine if resveratrol and EGCG also activate intracellular pathways other than the caspase pathway as seen in our study. Another study, Peritore et al focused on how EGCG and resveratrol affected the toxicity of SKNSH cells resulting from L-DOPA-induced hydrogen peroxide (34). They discovered that after 24 hours of pretreatment with 50 μM L-DOPA, 50 μM L-DOPA + 0-10 μM resveratrol and 50 μM L-DOPA + 0-100 μM EGCG, resveratrol and EGCG boosted the viability of SKNSH cells. Our conclusions are compatible with those of Peritore et al Sui et al examined the effect of resveratrol alone on hydrogen peroxide toxicity, subjecting human CRL 1730 umbilical vein endothelial cells to 100 M $H_2O_2$ for 24 hours after pre-treating them for two hours with resveratrol; the study revealed that resveratrol boosted cell viability by lowering the levels of intracellular caspase 3 (35). Our study showed compatible results to Sui et al's research in terms of lowering resveratrol levels in caspase 3.

In our study, EGCG increased cellular survival by reducing caspase 3 levels. In contrast to our findings, a study examining the effect of EGCG and resveratrol against severe toxicity in PC12 cells found no statistically significant effect of resveratrol on cell survival (36). The use of a different toxic material in our research may have shown resveratrol to have different effects on cell survival.

Konyalioglu et al conducted research utilizing embryonic neural stem cells in which 100 M hydrogen peroxide was given to ENSCS cells for 30 minutes. Hydrogen peroxide toxicity was treated with several dosages of resveratrol in concentrations ranging from 5 to 250 M for 30 minutes. The researchers discovered that resveratrol had protective effects against hydrogen peroxide toxicity by altering active catalase, glutathione peroxidase, nitric oxide synthetase, and nitric oxide (37). In our study, resveratrol showed a neuroprotective effect comparable to the results of Konyalioglu et al's study, where the same oxidant and antioxidant agents were used. However, our study used different and larger scale tests to obtain its results.

In summary, our study's results indicate that the increase in caspase 3 induced by $H_2O_2$ increased neuroblastoma cell apoptosis and decreased viability. EGCG and resveratrol pretreatment increased the survival rate of SH-SY5Y cells exposed to $H_2O_2$ and reduced caspase 3 levels. These results suggest that EGCG and resveratrol potentially prevent neurodegeneration associated with H2O2-induced oxidative stress (37).

## Conclusion

The antioxidant replacements EGCG and resveratrol exhibited protective action against hydrogen peroxide toxicity as a result of the research conducted on SH-SY5Y cells, which represent dopaminergic cell line characteristics. In order to increase cellular lifespan by lowering caspase 3 levels in neuronal cells, resveratrol and EGCG are two potential antioxidant compounds that can alleviate neurodegenerative disorders. Further studies will be required to examine in more detail the mechanisms of action of these two neuroprotective compounds, and in vivo research is required to examine their effects on the entire organism.

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