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# Protective Role of Corticosterone against Hydrogen Peroxide-Induced Neuronal Cell Death in SH-SY5Y Cells

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## Abstract

Stress breaks body balance, which can cause diverse physiological disorders and worsen preexisting diseases. However, recent studies have reported that controllable stress and overcoming from stress reinforce resilience to resist against more intense stress afterwards. In this study, we investigated the protective effect of corticosterone (CORT), a representative stress hormone against hydrogen peroxide ($H_2O_2$)-induced neuronal cell death and its underlying molecular mechanism in SH-SY5Y cells, a human neuroblastoma cell line. The decreased cell viability by $H_2O_2$ was effectively restored by the pretreatment with low concentration of CORT (0.03 μM for 72 h) in the cells. $H_2O_2$-increased expression of apoptotic markers such as PUMA and Bim was decreased by CORT pretreatment. Furthermore, pretreatment of CORT attenuated $H_2O_2$-mediated oxidative damages by upregulation of antioxidant enzymes via activation of nuclear factor erythroid 2-related factor 2 (Nrf2). These findings suggest that low concentration of CORT with eustressed condition enhances intracellular self-defense against $H_2O_2$-mediated oxidative cell death, suggesting a role of low concentration of CORT as one of key molecules for resilience and neuronal cell survival.

**Key Words:** Apoptosis, Eustress, Corticosterone, Nrf2, Antioxidant enzymes

## INTRODUCTION

Humans are constantly physically or psychologically stimulated. Stress is defined as physical and psychological conditions that disturb the homeostasis, leading to fear, anxiety, or tension (Sahin and Gumuslu, 2007; Samarghandian *et al*., 2017). In acute stress, the serum concentration of catecholamines increases when there is fear or anxiety, whereas in chronic stress, the hypothalamic-pituitary-adrenal (HPA) axis is activated, thereby increasing the secretion of glucocorticoids (GC) (Herman *et al*., 2016). Furthermore, the dysregulation of the HPA axis due to stress can be a risk factor for degenerative brain diseases, such as Alzheimer's disease and Parkinson's disease (Jiang *et al*., 2019).

A negative stress, so called distress, disrupts the balance of the body and causes pathological conditions and diseases (Lu *et al*., 2021). Oxidative stress is a representative intracellular mechanism that induces various diseases (Chen and Zhong, 2014; Zhang *et al*., 2015). GC secretion is increased by stress, which increases the secretion of excitatory glutamate, induces oxidative damage, and causes neuronal cell death (Madrigal *et al*., 2006; Samarghandian *et al*., 2017; Aalling *et al*., 2018).

In contrast, stress within the properly controlled range can give vitality to life and protect our health by moderately straining our bodies. Stress that helps our body and becomes a positive signal to have strong adaptability is called eustress (Lu *et al*., 2021). Eustress can enhance resilience from a macro perspective. Resilience is the opposite of vulnerability. 'Resilience' has been mainly used in positive psychology as a term to refer to the human ability to not only stand up again but even to become stronger in the face of severe life challenges.

From a cellular point of view, eustress is known to enhance resilience. According to the neurohormesis hypothesis, nerve cells respond to minor stress and activate the adaptive process to control and overcome stress, thereby resisting cell damage or disease. In fact, the long-term administration of a minimum dose of corticosterone (CORT) positively affected the development of hippocampal structure and function in the adrenalectomy mouse (C57BL/6) model (He *et al*., 2009).

In this study, we investigated the role and function of GC

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related to the enhancement of stress resilience. Therefore, we induced neuronal toxicity using $H_2O_2$ after pretreating low concentrations of CORT in SH-SY5Y cells. To investigate the effect and mechanism of action of CORT at low concentrations on $H_2O_2$-induced oxidative stress and neuronal cell death, cytotoxicity markers, oxidative damage, and neuronal cell death were sequentially examined.

## MATERIALS AND METHODS

### Materials

SH-SY5Y human neuroblastoma cells used in this study were purchased from American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and antibiotics (penicillin/streptomycin) were purchased from Gibco (Grand Island, NY, USA). Common reagents, including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR123) was provided by Invitrogen Co. (Carlsbad, CA, USA). Primary antibodies against of p53 upregulated modulator of apoptosis (PUMA), Bim, nuclear factor erythroid 2-related factor 2 (Nrf2), glutamylcysteine synthetase (GCS), manganese superoxide dismutase (MnSOD) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA , USA). Primary antibodies for 4-hydroxynonenal (4-HNE) and phospho-Nrf2 (p-Nrf2) were obtained from Abcam (Cambridge, UK). Anti-heme oxygenase-1 (HO-1) antibody was supplied by Enzo life sciences (Farmingdale, NY, USA).

### Cell culture

SH-SY5Y human-derived neuroblastoma cells were cultured using DMEM medium containing 10% FBS, penicillin (10,000 U/mL), and streptomycin (100 μg/mL) in an incubator at 37°C under 5% $CO_2$. The medium was replaced with fresh medium every 2 days.

### Cell viability

MTT assay was used to measure cell viability. SH-SY5Y cells were seeded at $4\times10^4$ cells/300 μL per well in a 48-well culture plate. When the cells were stably attached after 24 h, they were treated with CORT and $H_2O_2$. After culturing the cells for 24 h after treatment with $H_2O_2$, MTT (final concentration 1 mg/mL) reagent was added and cells were incubated at 37°C under 5% $CO_2$ for 2 h. After removing the supernatant, 200 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan. Then, absorbance at 540 nm was measured using an ELISA reader (Emax, Molecular Devices LLC, San Jose, CA, USA). Cell viability (%) was calculated based on the absorbance of the control group cultured in medium without reagents.

### Western blotting

The protein amount was quantified using BCA reagent, and 35 μg of protein was electrophoresed using 10–12% SDS-PAGE. After transferring proteins from the developed gel to a PVDF membrane, the membrane was blocked with 5% fat-free dry milk-phosphate buffered saline containing 0.1% Tween-20 (PBST). The membrane was incubated with the primary antibodies in 5% fat-free dry milk-PBS overnight, then washed thrice for 10 min each with PBST solution. Following incubation with horseradish peroxidase conjugated-secondary antibodies in 5% fat-free dry milk-PBS for 1 h, it was washed thrice for 10 min each with PBST solution. The membrane was reacted with ECL reagent for 1 min and images were captured using chemiluminescent Immunoblotting equipment.

### Measurement of reactive oxygen species

SH-SY5Y cells were plated at a density of $6\times10^4$ cells/300 μL in 48-well plates or $1\times10^5$ cells/500 μL in 4-well chamber slide and treated with $H_2O_2$ in the presence or absence of CORT. After 24 h-treatment, cells were incubated with 25 μM DHR123 solutions for 15 min at 37°C, respectively. For 48-well plates, the cells were washed with PBS and solubilized with DMSO and then the relative fluorescence intensity was measured by a microplate reader (Infinite M200 Pro, Tecan Group Ltd., Männedorf, Switzerland) with excitation at 485 nm and emission at 535 nm.

### Statistical analysis

All experimental results in this study are expressed as mean ± standard deviation. For statistical significance, analysis of variance (ANOVA) was performed using the SPSS program (version 24.0, IBM, Chicago, IL, USA), and the significant difference between the measured mean values was post-tested with the Turkey test. Statistical significance was set at $p<0.05$.

## RESULTS

### Effects of CORT on cell viability

SH-SY5Y cells were treated with various concentrations of CORT to determine the concentrations of CORT which could induce eustress on neuronal cells. Cell viability was also measured using MTT assay. At 1 μM concentration of CORT, the viability was 20.7% lower than that of the control group. There was no substantial change in cell viability at CORT concentrations of up to 25 μM, but there was a tendency for cell viability to decrease at concentrations between 25-100 μM. At 100 μM, the viability was 36.12% lower than that of the control group. In cases where the cell viability was decreased by more than 20%, compared with control group, it was judged as excessive stress (distress). However, when low concentrations of CORT (0.003 μM, 0.01 μM, and 0.03 μM) were treated, the decrease in cell viability was only up to 10% from the control group (8.86%, 8.84%, and 10.48%, respectively) (Fig. 1). Therefore, it was considered as eustress.

### Protective effect of CORT against $H_2O_2$-induced neurotoxicity.

To examine the effect of low concentrations of CORT on $H_2O_2$-induced neuronal toxicity, SH-SY5Y cells were incubated with two concentrations (0.003 μM and 0.03 μM) of CORT that induce eustress. MTT assay was performed 24 h after treatment with $H_2O_2$ (250 μM). As a result, the viability was 59.75% in the group not treated with CORT. However, substantial protective effects were observed in the CORT-treated group, increasing the cell viability to 76.97% at 0.003 μM and 98.34% at 0.03 μM CORT, respectively (Fig. 2).

### Protective effect of CORT against $H_2O_2$-induced apoptosis

To investigate the protective effect of CORT against $H_2O_2$-induced apoptosis, the expression of PUMA and Bcl-2-like

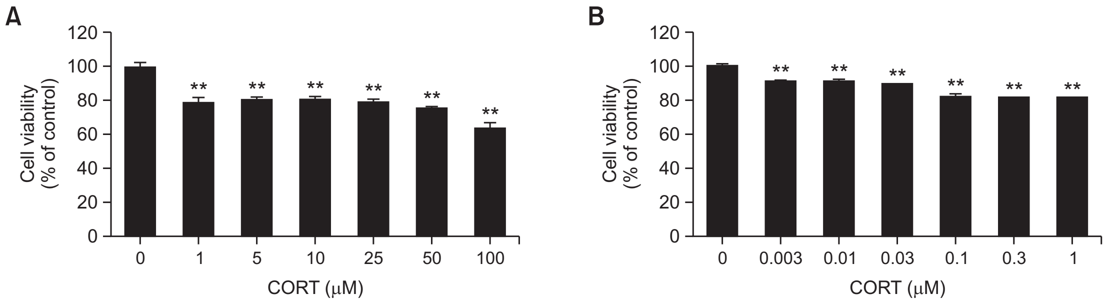


**Fig. 1.** Effect of CORT on cell viability in SH-SY5Y cells. The cells were treated with various concentration of CORT for 24 h. (A) High concentration groups were treated with 1, 5, 10, 25, 50 and 100 μM CORT. (B) Low concentration groups were treated with 0.003, 0.01, 0.03, 0.01, 0.3 and 1 μM CORT. Cell viability was determined by using MTT assay. Data were presented as mean ± SEM (n=3), ***p*<0.01 vs. control group.

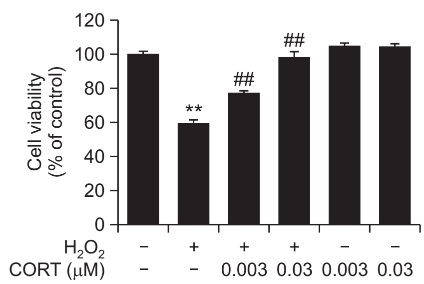


**Fig. 2.** Effect of CORT against $H_2O_2$-decreased cell viability in SH-SY5Y cells. The cells were pretreated with 0.003 and 0.03 μM of CORT for 72 h and then incubated with or without 250 μM $H_2O_2$ for 24 h. Cell viability was determined by using MTT assay. Data were presented as mean ± SEM (n=3), ***p*<0.01 vs. control group; ##*p*<0.01 vs. $H_2O_2$-treated group.

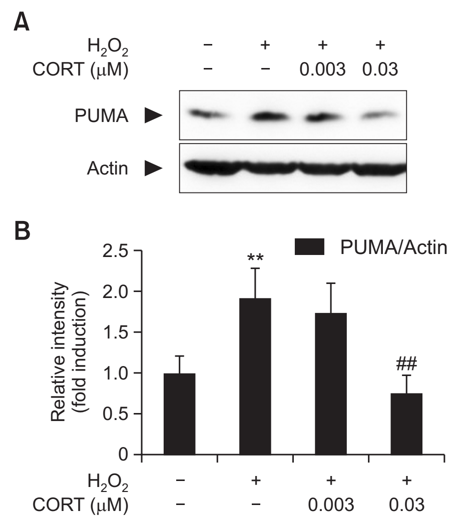


**Fig. 3.** Effect of CORT on $H_2O_2$-induced PUMA expression in SH-SY5Y cells. The cells were pretreated with 0.003 and 0.03 μM CORT for 72 h and then incubated with or without 250 μM $H_2O_2$ for 24 h. PUMA expression was determined by western blot (A), and presented a relative intensity of PUMA/Actin (B). Data were expressed as mean ± SEM (n=3), ***p*<0.01 vs. control group; ##*p*<0.01 vs. $H_2O_2$-treated group.

protein 11 (also known as Bim), was measured by western blotting. PUMA is known to induce apoptosis by binding to the anti-apoptotic protein Bcl-2 and interfering with its function. Bim induces apoptosis in response to various apoptotic-inducing signals. SH-SY5Y cells were incubated with 0, 0.003, and 0.03 μM concentrations of CORT for 72 h. In 24 h after treatment with $H_2O_2$ (250 μM), protein expression was measured by western blot analysis. The expression of PUMA was significantly higher in the cells treated with only $H_2O_2$ compared with that in the control group, but the expression was slightly lower when treated with 0.003 μM CORT. In the group treated with 0.03 μM CORT, PUMA expression was reduced, similar to that in the control group (Fig. 3). Similar to PUMA, the expression of Bim was increased in cells treated with only $H_2O_2$, but slightly decreased when treated with 0.003 μM CORT. In the group treated with 0.03 μM CORT, the expression level was reduced to similar to that of the control group (Fig. 4).

### Inhibitory effect of CORT on oxidative damages

To examine the inhibitory ability of CORT on oxidative damages, 4-HNE expression was measured by using western blot analysis. 4-HNE is produced during cellular lipid peroxidation. It is known that much more 4-HNE is produced when oxidative stress is applied. SH-SY5Y cells were cultured with CORT for 3 days and then treated with 250 μM $H_2O_2$. After culturing for 24 h, 4-HNE expression was measured. Lipid peroxidation was increased in cells treated with only $H_2O_2$ but slightly decreased when treated with 0.003 μM CORT. In the case of 0.03 μM CORT treatment, the expression level was reduced to similar to that of the control group (Fig. 5A, 5B). In addition, fluorescence of DHR123 was measured to detect reactive oxygen species (ROS) such as peroxide and peroxynitrite. Treatment of SH-SY5Y cells with 250 μM $H_2O_2$ led to an increased production of ROS as shown by the intense fluorescence inside cells, which was decreased by CORT (0.003, 0.03 μM) pretreatment (Fig. 5C).

### Antioxidant mechanism of CORT

To elucidate the mechanism of action of CORT, which has a protective action against oxidative neuronal cell death, protein expression of representative intracellular antioxidant enzymes was measured by western blot analysis. Various antioxidant defense mechanisms act *in vivo* to remove excessively generated ROS. Examples of these are GCS, HO-1, and MnSOD, including enzymatic or non-enzymatic antioxidant proteins and

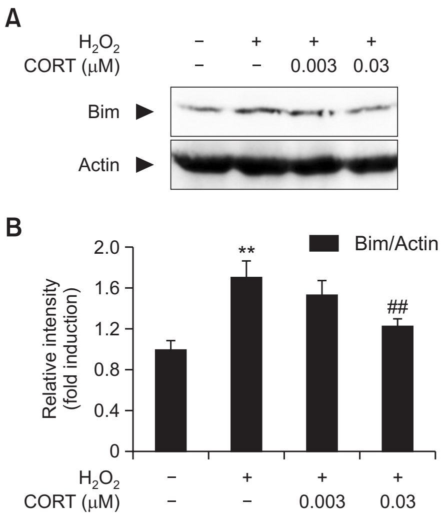


**Fig. 4.** Effect of CORT on $H_2O_2$-induced Bim expression in SH-SY5Y cells. The cells were pretreated with 0.003 and 0.03 μM CORT for 72 h and then incubated with or without 250 μM $H_2O_2$ for 24 h. Bim expression was determined by western blot (A), and presented a relative intensity of Bim/Actin (B). Data were expressed as mean ± SEM (n=3), $^{**}p<0.01$ vs. control group; $^{\#\#}p<0.01$ vs. $H_2O_2$-treated group.

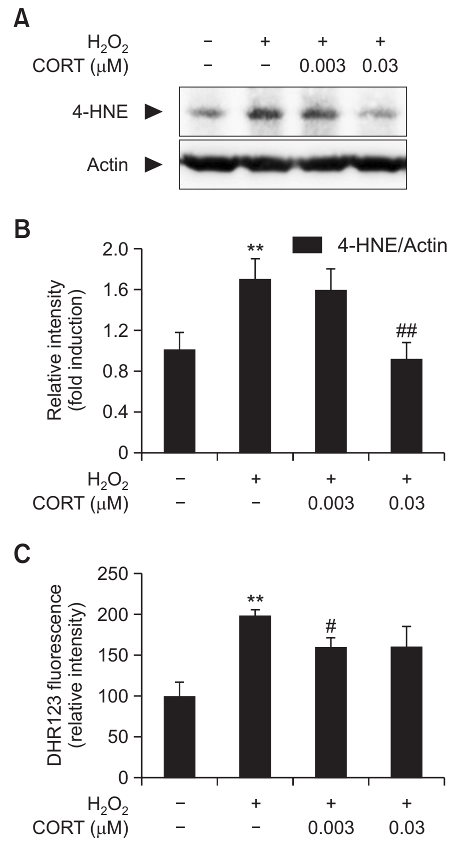


**Fig. 5.** Effect of CORT against $H_2O_2$-induced 4-HNE expression and ROS production in SH-SY5Y cells. The cells were pretreated with 0.003 and 0.03 μM CORT for 72 h and then incubated with or without 250 μM $H_2O_2$ for 24 h. 4-HNE expression was determined by western blot assay (A) and presented a relative intensity of 4-HNE/Actin (B). Intracellular ROS was determined by using DHR 123 fluorescence dye (C). Data were expressed as mean ± SEM (n=3), $^{**}p<0.01$ vs. control group; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. $H_2O_2$-treated group.

genes. SH-SY5Y cells were cultured with CORT for 3 days and then treated with $H_2O_2$. After culturing for 24 h, proteins were isolated, and the expression of representative antioxidant enzymes GCS, HO-1, and MnSOD was measured. In the cells with no treatment of CORT, all the measured antioxidant enzymes showed only slight increase, whereas their expression was significantly increased when CORT was treated. Particularly there was a remarkable difference between HO-1 and MnSOD with or without CORT. In the case of GCS, expression was increased at the 0.03 μM of CORT (Fig. 6).

### Upstream antioxidant defense mechanisms

Next, a series of experiments were conducted to examine the activation of Nrf2 to elucidate the molecular mechanism of action by which low concentration CORT increases the expression of these antioxidant enzymes. In general, Nrf2 is present in the cytosol by forming a complex with Kelch-like ECH-associated protein 1 in an inactive state. However, when activated by phosphorylation, it moves into the nucleus and binds to the ARE binding site, thereby regulating the expression of various detoxification and antioxidant enzymes. When cells were cultured for 72 h after simultaneously treating CORT and $H_2O_2$, there was a clear difference in p-Nrf2 expression level depending on the presence or absence of CORT. There was a slight change in expression when only $H_2O_2$ was treated compared with the control group. However, the expression level was recovered when treated with a low concentration (0.003 μM) of CORT, which was maintained even up to 0.03 μM CORT (Fig. 7).

## DISCUSSION

The SH-SY5Y neuronal cell line appeared to be resistant to stress in the presence of low concentrations of CORT between 0.003 μM and 0.03 μM. When 250 μM $H_2O_2$ was used as a condition to induce oxidative stress, the protective effect was observed at low concentrations of CORT for an extended period. The concentrations of CORT that were toxic in various cell experiments ranged from 1 to 5 mM.

In contrast, according to several previous studies, mild stress and chronic multiple stress in aged mice and rats showed positive effects in enabling learning and memory through increased expression of Fyn and brain-derived neurotrophic factor, hippocampal neurogenesis, and synaptic plasticity (Li *et al*., 2007). In addition, stroke-induced memory impairment was recovered by alleviating hippocampal damage by mild restraint stress and treatment with CORT (Faraji *et al*., 2009). In addition, mild restraint stress increased the level of CORT and activity of glucocorticoid receptor in CBA male mice, thereby protecting acoustic trauma (Tahera *et al*., 2006).

As a result of investigating the mechanisms underlying oxidative stress induction and neuronal cell death by $H_2O_2$ following pretreatment with low concentrations of CORT, pro-apoptotic signal markers and oxidative stress induced by $H_2O_2$ were attenuated when low concentrations of CORT were pre-

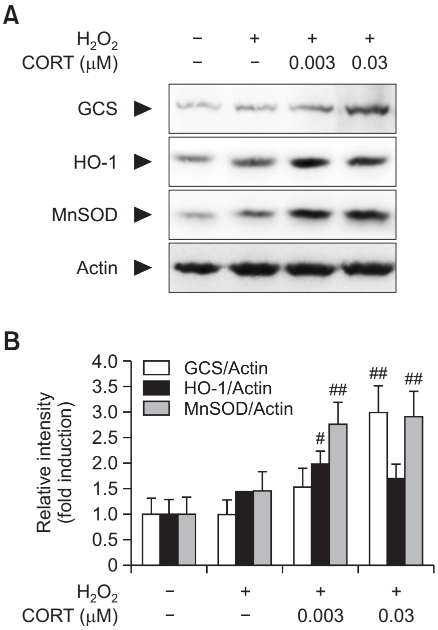


**Fig. 6.** CORT-induced expression of antioxidant enzymes in SH-SY5Y cells. The cells were pretreated with 0.003 and 0.03 μM CORT for 72 h and then incubated with or without 250 μM $H_2O_2$ for 24 h. Expression levels of HO-1, GCS and MnSOD were measured by western blot assay (A) and presented a relative intensity of HO-1/Actin, GCS/Actin and MnSOD/Actin (B). Data were expressed as mean ± SEM (n=3), $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. $H_2O_2$-treated group.

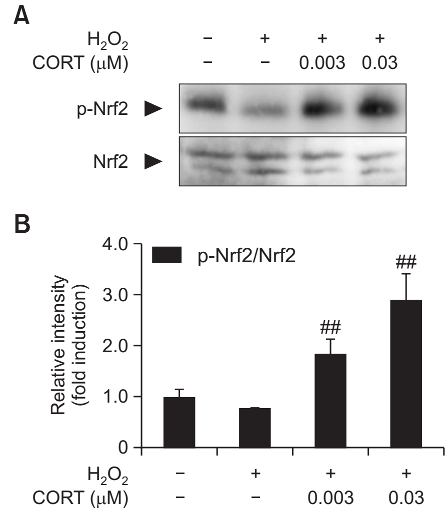


**Fig. 7.** CORT-recovered activation of Nrf2 in SH-SY5Y cells The cells were pretreated with 0.003 and 0.03 μM CORT for 72 h and then incubated with or without 250 μM $H_2O_2$ for 24 h. Phosphorylation of p-Nrf2 at Ser40 were determined by western blot assay (A) and calculated a relative intensity of p-Nrf2/Nrf2 (B). Data were presented as mean ± SEM (n=3), $^{\#\#}p<0.01$ vs. $H_2O_2$-treated group.

treated. Previous studies have shown that $H_2O_2$ can activate various apoptosis signals in neuronal cells (McNeill-Blue *et al*., 2006; Ye *et al*., 2014; Gao *et al*., 2021). In particular, $H_2O_2$ activated PUMA and Bim in PC12 cells, which were inhibited by the antioxidant vitamin C. The increased expression of cleaved caspase-3, nuclear factor kappa-light-chain-enhancer of activated B cells, and apoptosis due to myocardial injury were decreased by low concentration of hydrocortisone in an LPS-induced early septic shock SD rat model (Liao *et al*., 2020).

Meanwhile, $H_2O_2$ attacks lipids, proteins, and genes, which are macromolecules *in vivo* in neurons, and induces cell death through lipid peroxidation, protein oxidation, and oxidative damage of genes. In *in vivo* and *in vitro* studies, it has been reported that high concentrations of CORT can increase neurotoxicity and apoptosis through reactive oxygen species generation. However, it is known that CORT in the appropriate range of concentrations has a protective effect against oxidative stress induced by single nucleotide polymorphisms.

In particular, oxidative stress was reduced by treatment with low concentrations of CORT due to the increased activity of the induced transcription factor Nrf2 and the increased expression of the antioxidant enzymes HO-1, GCS, and MnSOD. When SH-SY5Y cells were treated with $H_2O_2$, Nrf2 activity and antioxidant enzyme expression were reduced when cytotoxicity occurred, but the cytotoxicity of $H_2O_2$ was recovered by various phytochemicals that activate Nrf2.

In conclusion, this study demonstrated that low concentrations of CORT can act as eustress, which gave SH-SY5Y resilience and protection against neuronal toxicity and death induced by $H_2O_2$, which is distress. In particular, CORT suppressed oxidative stress by increasing the expression of antioxidant enzymes, which appears to inhibit apoptosis by reducing the expression of proapoptotic proteins. CORT increased the expression of representative antioxidant enzymes HO-1, GCS, and MnSOD through phosphorylation of Nrf2 as an upstream target.

## CONFLICT OF INTEREST

The authors have no conflicts of interest.

## ACKNOWLEDGMENTS

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