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EXPERIMENTAL STUDY

Caffeic acid prevents hydrogen peroxide-induced oxidative damage in SH-SY5Y cell line through mitigation of oxidative stress and apoptosis

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ABSTRACT

AIM: The aim of this study was to examine the potential ameliorative effects of caffeic acid (CA) on hydrogen peroxide (H_2O_2)-induced neurodegeneration in a human SH-SY5Y cell line, as well as possible mechanisms involved.

METHOD: Cell proliferation was evaluated by WST-1 assay. The apoptotic index was calculated by TUNEL Assay. Antioxidant parameters were studied by measuring reactive oxygen species (ROS), lipid peroxidation (LPO) levels, and catalase (CAT) activity. The mRNA expression levels of apoptotic and anti-apoptotic genes were studied by qRT-PCR

RESULTS: In this study, the pre-treatment with CA significantly suppressed H_2O_2-stimulated cell death and apoptosis in SH-SY5Y cell line. The mechanism by which CA pretreatment protected the cells from oxidative injury includes the decrease in ROS and LPO levels, increase in CAT activity, down-regulation of mRNA levels of Bax, cytochrome c, cas-3, cas-8, and p53, and up-regulation of anti-apoptotic Bcl-2 gene.

CONCLUSION: These results reveal that CA plays a role in the protection from oxidative injury-triggered apoptosis, which makes CA a likely therapeutic compound for treatment or prevention of neurodegenerative disorders associated with oxidative injury *(Fig. 5, Ref. 35)*. Text in PDF *www.elis.sk*

KEY WORDS: caffeic acid; apoptosis; oxidative stress; cas-3; reactive oxygen species.

Introduction

The frequency of neurodegenerative diseases has been growing owing to the increase in older population. Artificial neuroprotective medicines used for treating the neurodegenerative disorders have been accompanied with some adverse effects. Therefore, more consideration has been attributed to the neuroprotective potential of using natural substances that have fewer adverse effects (1).

Neurodegenerative disorders are associated with DNA mutation in mitochondria and oxidative injury which occurs when reactive oxygen species (ROS) are produced in mitochondria (2–5). Superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) are among the key ROS. When the levels of ROS overcome the cellular antioxidant protection machinery, oxidative stress occurs, which results in ROS-intermediated destruction of lipids and proteins contributing to neurodegenerative diseases and carcinogenesis (6). For that reason, cells must frequently regulate the amount of ROS and prevent their accumulation. These disorders might be reversed with the aid of antioxidant supplementation with considerable success. Several studies have been carried out to develop efficient and non-toxic antioxidants that could be beneficial for the treatment and prevention of neurological disorders (7).

Antioxidants co-operatively perform against free radicals to fight their destructive effects on crucial macro biomolecules and eventually body tissues. Depending on their reaction to the free-radical attack, they have been categorized as first, second, third and even fourth-line defense antioxidants. The role and efficiency of the first-line defense antioxidants, which contain SOD, CAT and GPX, is essential and obligatory in the whole defense scheme of antioxidants. A great deal of studies have been conducted regarding antioxidants, their importance in prohibiting oxidative damage and associated cellular injury, as well as on the vital role of SOD, CAT and GPX (8–11). Caffeic acid (CA) is among the major hydroxycinnamic acid derivatives and represents a potent antioxidant activity (12).

The aim of this study was to examine the potential ameliorative effects of CA on H_2O_2-induced neurodegeneration in human neuronal cell line, as well as possible mechanisms involved.

Material and Method

Cell culture and treatment

SH-SY5Y cells were grown at 37° in a humidified 5% CO_2, in Dulbecco's modified Eagle's medium complemented with 10% fatal bovine serum, 1% penicillin-streptomycin as previously explained (13).

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Experimental groups

The neuron cells were separated in three groups. In the control group, the cells were grown in complete medium. In H_2O_2 group, the cells were grown in the presence of 0.8 mM H_2O_2 for 12 h. In CA+H_2O_2 pre-treatment group, the cells were pre-incubated with CA (0.1 mM) for 3 h, and 0.8 mM H_2O_2 was added for 12 h.

Cell proliferation assay

Cytotoxicity experiments were carried out by water-soluble tetrazolium-1 (WST-1) cell viability assay kit as described in kit procedure (Clontech Laboratories).

Lipid peroxidation (LPO) assay

In order to evaluate lipid peroxidation level in H_2O_2-induced SH-SY5Y cells, a slight modification of previously published procedure was used (14).

Intracellular ROS assay

Cellular ROS generation was assessed by use of 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) assay kit (Abcam, MA, USA), as previously defined (15).

Catalase (CAT) activity and total antioxidant status (TAS)

The experiments were carried out following the protocols supplied with the catalase assay kit (Elabscience) and human TAS ELISA kit (SunRed).

TUNEL assay

The assay was performed following the procedure supplied with the ApopTag kit (Milipore).

QRT-PCR analysis

The expression levels of the genes studied within this research were investigated by quantitative real-time gene expression assay kit (Jena Bioscience). The expression levels of *Bax, Bcl-2, cytochrome c, p53, cas-8 cas-3*, and glyceraldehyde-3-hosphate dehydrogenase (*GAPDH),* as a housekeeping gene, were analyzed as previously explained (15).

Caspase-3 staining

Immuno-histochemical staining experiments were carried out by use of UltraVision LP Large Volume Detection System (Santa Cruz Biotechnology) kit protocol.

Statistics

All experiments were repeated for at least three times. The statistical analysis and comparable data groups were assessed using GraphPad Prism 5 by one-way ANOVA Newman-Keuls post-hoc test; $p < 0.05$ was considered significant.

Results

Cell proliferation assay

The cells were exposed to 25, 50, 100, 200, 400 and 800 µM H_2O_2 for 12 h (24 h data not given) to decide the concentration and time of H_2O_2 exposure needed to produce toxicity on SH-SY5Y cell line. The cell toxicity was evaluated by WST-1 assay. The exposure to H_2O_2 for 12 h reduced the cell proliferation in a dose-dependent and timely manner. As illustrated in Figure 1A, 800 µM H_2O_2 induced remarkable reductions in cell survival while CA treatment (100 µM) protected the cells from H_2O_2-induced injury (Figs 1A, 1B).

Detection of cellular ROS and LPO level and CAT activity

The effects of CA pre-treatment on H_2O_2-triggered ROS production in SH-SY5Y cells were assessed. The results revealed that H_2O_2 exposure led to a remarkable rise in ROS production; however, 100 µM CA pre-treatment significantly reduced ROS production (Fig. 2A). To assess whether CA contributed to the protection of neuron cells from H_2O_2-induced injury, LPO assay was performed using 100 µM CA. As shown in Figure 2B, the level of LPO in neuron cells from H_2O_2 group considerably increased while CA pre-treatment reduced LPO levels, as compared with SH-SY5Y cells from H_2O_2 group. The CAT activity was significantly decreased in SH-SY5Y cells from the H_2O_2 group, as compared to the control group, while CA pre-treatment increased the CAT activity (Fig. 2C).

TUNEL Assay

The effect of CA on preventing apoptosis triggered by H_2O_2 in neuron cells was also evaluated. The results revealed that H_2O_2 induced apoptosis by causing breaks in the DNA strand and increasing AI, as determined by TUNEL assay. The pretreatment with CA significantly inhibited the H_2O_2-induced apoptosis (Fig. 3).

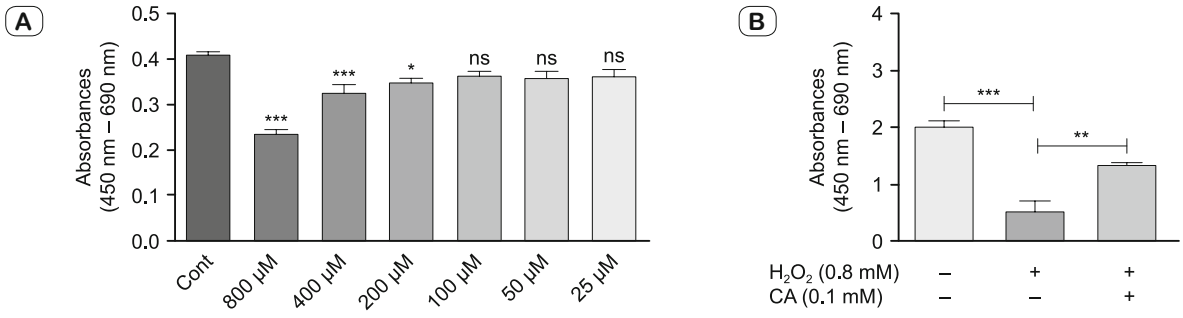

Fig. 1. Effects of H_2O_2 (A) and CA+ H_2O_2 on neuronal cell proliferation (B). Experimental data are presented as mean ± SEM ($n \geq 3$); *** $p < 0.001$ points out statistically significant differences between control and other groups; ns: not significant.

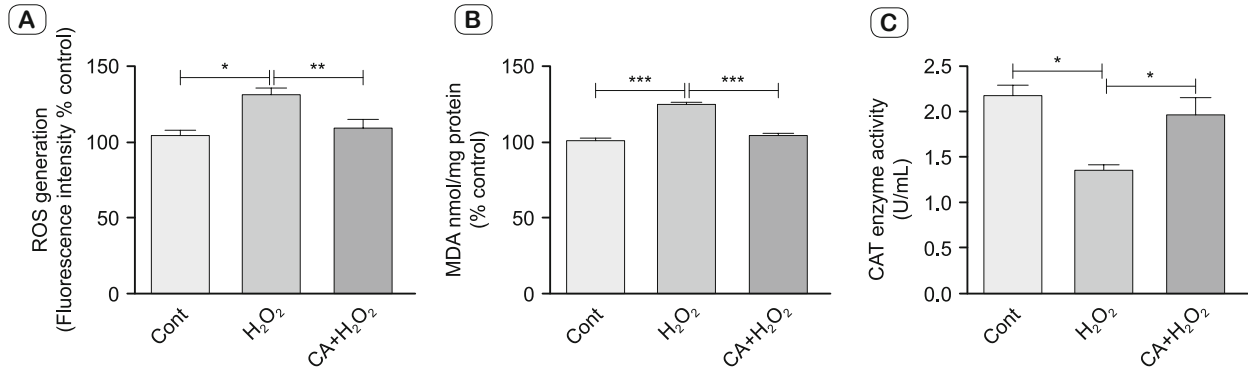

Fig. 2. The effect of CA on H_2O_2-induced ROS generation (A), MDA level (B) CAT activity (C) in SH-SY5Y cells. Experimental data are given as mean ± SEM (n ≥ 3); *** p < 0.001, ** p < 0.01, * p < 0.05 point out statistically significant differences between control and other groups.

Immunohistochemical staining for caspase-3

To approve whether H_2O_2-induced cell toxicity occurred owing to the induction of apoptotic pathway, the activation of caspase-3, an important executioner of apoptosis, was determined. As demonstrated in Figure 4, caspase-3 was considerably activated in SH-SY5Y cells by the exposure to H_2O_2 while the pretreatment with CA remarkably reduced the caspase-3 activation.

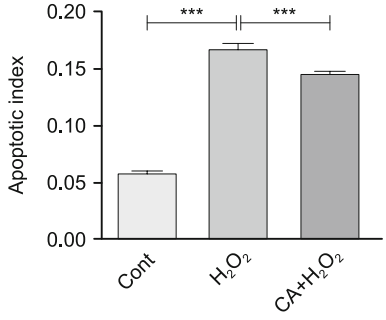

Fig. 3. The effect of H_2O_2 and CA+H_2O_2 treatment on apoptosis in SH-SY5Y, as compared to the control group. AI was calculated according to the TUNEL assay instructions. Experimental data are given as mean ±SEM (n ≥ 3); *** p < 0.001 points out statistically significant differences between control and other groups.

QRT-PCR analysis

To test whether the expression levels of apoptotic and pro-apoptotic genes were affected by H_2O_2 and CA treatment, the mRNA levels of *Bax*, *Bcl-2*, *cytochrome c*, *p53*, *cas-8* and *cas-3* were measured by qRT-PCR. As illustrated in Figure 5 (A–F), the mRNA levels of *Bax*, *cytochrome c*, *p53*, *cas-8* and *cas-3* were dramatically upregulated while mRNA levels of anti-apoptotic *Bcl-2* were downregulated after H_2O_2 exposure. The pretreatment with CA considerably reversed the mRNA levels of these genes.

Discussion

Neurodegenerative diseases have been characterized by progressive deterioration of the neuronal function as a result of degeneration of synapses and axons eventually leading to the death of neuron cells. More profound comprehension of the mechanisms underlying the altered cellular homeostasis and neurodegeneration is crucial for the development of efficient treatments for the diseases (16). Oxidative stress which happens when the cellular antioxidant defense machinery is overcome by excessive ROS levels might influence the cell viability and survival through activating metabolic cell signaling pathways. Oxidative injury is confirmed as a contribution to the pathology of many neurological disorders

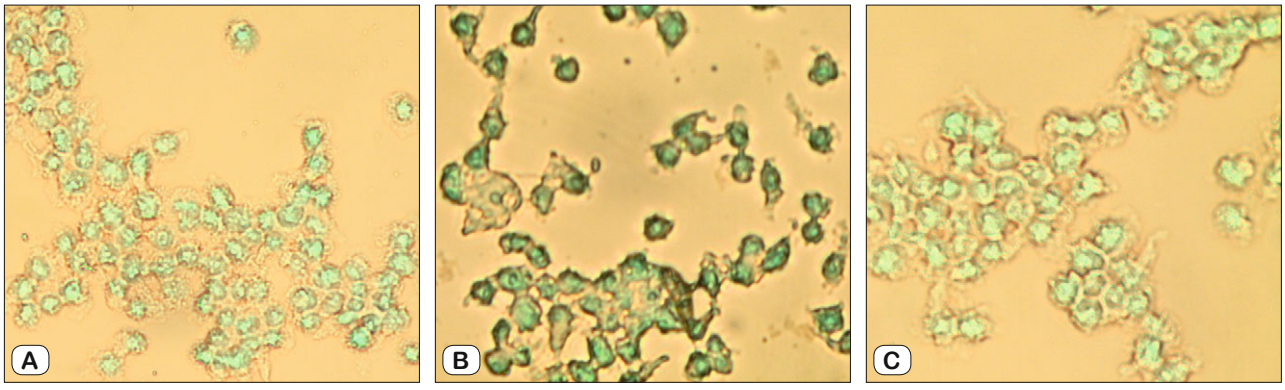

Fig. 4. The effect of H_2O_2 (B) and CA+H_2O_2 treatment (C) on apoptosis related active caspase-3 in SH-SY5Y cells, as compared to the control group (A). Caspase-3-expressing cells are characterised by their dark brown colour.

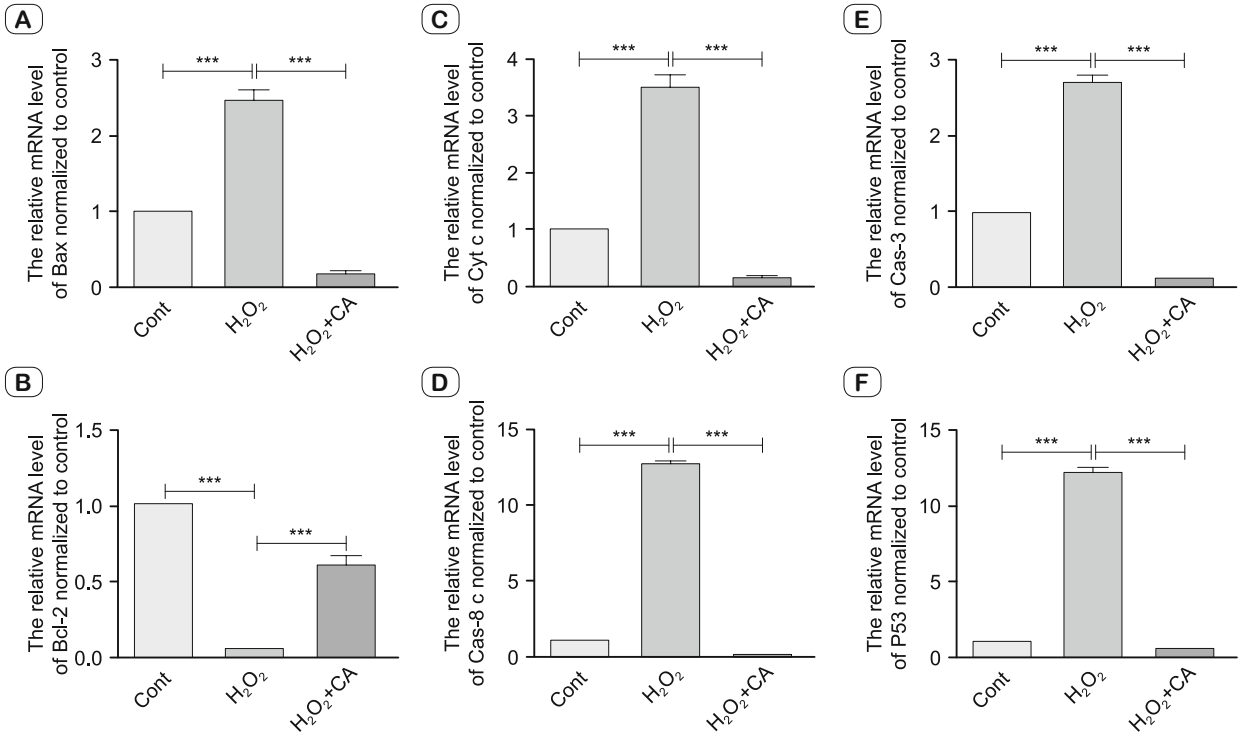

Fig. 5. The effect of H_2O_2 and CA+ H_2O_2 on the level of apoptosis-related gene expressions in SH-SY5Y cells. The cells were treated as stated in experimental groups. The mRNA levels of pro-apoptotic Bax (A), anti-apoptotic Bcl-2 (B), apoptotic cytochrome c (C), cas-8 (D), cas-3 (E) and p53 (F) were measured by using QRT-PCR analysis. Experimental values are expressed as mean ± SEM ($n \geq 3$); *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ indicate statistically significant differences between control and other groups; ns: not significant.

(17–19). ROS have been demonstrated to provoke apoptotic cell death in endothelial vascular cells, glial and neuron cells (20–22). H_2O_2 has been frequently used to create *in vitro* models of oxidative injury by triggering excessive generation of ROS through targeting mitochondria, which results in mitochondrial dysfunction (23). A reasonable approach to maintaining cellular redox balance and protecting against oxidative injury lies in eliminating the excessive levels of ROS or inhibiting their production by antioxidants (24, 25). The phenolics that include CA can scavenge the free radicals either directly or via a sequence of coupled reactions with enzymatic antioxidants such as CAT (26–28). Given its reported properties as an antioxidant for oxidative injury, CA can be a motivating candidate to be examined in neurodegenerative models. H_2O_2, as an inducer of oxidative damage, revealed antioxidant activity of CA in neuron cells. As predicted, the pretreatment of cells with CA caused resistance to H_2O_2 toxicity, as shown by increased cell viability. The effect of CA on intracellular ROS and LPO levels after H_2O_2 treatment was also investigated. H_2O_2 triggered cellular oxidative injury as measured by ROS level, while the *in vitro* pre-treatment of neuron cells with CA led to elimination of H_2O_2-induced cellular toxicity. In the meantime, the *in vitro* pretreatment of cells with CA decreased the level of cellular LPO. The catalytic activity of CAT was noticeably reduced in neuron cells from H_2O_2 group, as compared to the normal cells, while the CA pretreatment increased the CAT activity. The decrease in oxidative stress associates with the rise in cell viability observed in neuron cells pretreated with CA, which supports the hypothesis that CA could significantly protect SH-SY5Y cells from H_2O_2-provoked cell apoptosis.

Several biochemical and physiological studies have been carried out to verify the connection between oxidative damage and apoptosis (29–31). H_2O_2 could possibly trigger apoptosis directly, while the presence of antioxidants might inhibit this effect (32). To examine whether H_2O_2 caused apoptosis in neurons, the AI was calculated via TUNEL assay. The protective effect of CA pretreatment against neuron apoptosis was studied as well. As shown in Figure 3, H_2O_2 treatment considerably increased the apoptotic index, while CA pretreatment remarkably decreased the apoptotic cell death, thus suggesting the protective role of CA in H_2O_2-triggered apoptosis in neuron cells.

The apoptotic pathway is accompanied with sequences of biological changes such as in caspase-3 level, a crucial executioner of neuronal apoptosis (33). The tumor suppressor gene *p53*, the activation of which is reported to trigger apoptosis in various cells including neurons, is an important regulator of cell stress (34). *Bax* is located in the cytoplasm but translocates to the mitochondrion after cellular death signaling, thus promoting the release of cytochrome c. In the extrinsic pathway, caspase-8 is shown to have the main role in activating caspase-3 (35). In this research, the treatment with H_2O_2 led to the rise in expression of *Bax*, *cytochrome c, cas-8, cas-3,* and *p53* and to a decrease in mRNA levels of anti-apoptotic *Bcl-2*, while their expression levels were

decreased by pretreatment with CA, which indicates that CA plays a role in prevention from H_2O_2-induced apoptosis in neuron cells.

Overall, the pretreatment with CA noticeably reduced the H_2O_2-induced cell toxicity and apoptosis. The machinery, by which CA pretreatment protected SH-SY5Y cells from oxidative damage, covers the reduction in ROS and LPO levels, increase in CAT enzyme activity, downregulation of expression of *Bax*, *cytochrome c*, *cas-3*, *cas-8*, *p53* and upregulation of anti-apoptotic *Bcl-2*. These results revealed that CA has a role in protection from oxidative injury-triggered apoptotic pathway, which makes CA a possible therapeutic compound in the treatment of or prevention from neurodegenerative disorders associated with oxidative injury.

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