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A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

**Coffee and chlorogenic acid inhibit hydrogen peroxide induced  
apoptosis in primary cortical neurons**

일차신경세포에서 커피 및 클로로젠산의 과산화수소로  
유도되는 세포사멸 억제효능

**By**

**Siyoung Lee**

**Department of Agricultural Biotechnology**

**Seoul National University**

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## **Abstract**

Oxidative injury has been linked causally to a variety of neurodegenerative diseases. Oxidative injury is mediated by reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ). Epidemiological studies have suggested that coffee may be neuroprotective, but their biological mechanisms are poorly understood. In this study, i investigated the protective effects of caffeinated coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid (5-O-caffeoylquinic acid), which is present in both caffeinated and decaffeinated coffee, against apoptotic cell death. I conformed neuroprotective effect of caffeinated coffee, decaffeinated coffee and chlorogenic acid thorough DAPI staining which identified  $H_2O_2$ -induced apoptotic nuclear condensation in neuronal cells. Caffeinated coffee, decaffeinated coffee, or chlorogenic acid also blocked the  $H_2O_2$ -induced down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-XL while

blocking H<sub>2</sub>O<sub>2</sub>-induced pro-apoptotic cleavage of caspase-3 and pro-poly (ADP-ribose) polymerase. I also found that caffeinated coffee, decaffeinated coffee, and chlorogenic acid induced the expression of NADPH:quinine oxidoreductase 1 (NQO1) in neuronal cells, suggesting that these substances protect neurons from H<sub>2</sub>O<sub>2</sub>-induced apoptosis by up-regulation of this antioxidant enzyme. The neuroprotective efficacy of caffeinated coffee was similar to that of decaffeinated coffee, indicating that active compounds present in both caffeinated and decaffeinated coffee, such as chlorogenic acid, may drive the effects.

**Key Words: Caffeinated coffee; decaffeinated coffee; chlorogenic acid; NADPH:quinine oxidoreductase 1 (NQO1); primary cortical neuron; hydrogen peroxide; apoptosis;**

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## **I. Introduction**

The beneficial effects of coffee on brain health have been investigated, primarily focusing on its well-known component caffeine because of its permeability across the blood–brain barrier and the psychoactive stimulating effects [1]. Several studies have reported that consumption of coffee or caffeine in both young and adult is associated with better cognitive performance [2, 3]. Other studies have suggested that coffee and caffeine may have protective effects against Alzheimer’s disease and Parkinson’s disease [4, 5]. On the other hand, a recent report suggested that decaffeinated coffee can also provide protection in *Drosophila* models of neurodegenerative diseases, suggesting that active compounds in coffee other than caffeine may also drive the effects [6].

Coffee is a complex chemical mixture consisting of a number of bioactive compounds. The chemical composition of coffee depends mainly on the variety of the coffee with slight variations made by agroclimatic

conditions, agricultural practices, processing, and storage [7]. On average approximate composition of coffee, chlorogenic acids (i.e., caffeoylquinic acids, feruloylquinic acids, dicaffeoylquinic acids, and, in smaller amounts, p-coumaroylquinic acids and their derivatives), are major phenolic phytochemicals found in both caffeinated and decaffeinated coffee [8]. Coffee contains approximately 7–9% polyphenolic chlorogenic acids but only 1% caffeine [7, 9, 10]. Therefore, studying the effect of chlorogenic acids may be necessary to fully understand the neuropharmacological effects of caffeinated and decaffeinated coffee.

Oxidative injury has been linked causally to a variety of neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and conditions such as ischemia and excitotoxicity [11-14]. Oxidative damage is mediated by reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion radical ( $O_2^-$ ), and hydroxyl radical (OH) [12, 15]. For example,  $H_2O_2$  is involved in the production of highly reactive hydroxyl radicals via

Fenton's reaction and can react with nearly all cellular macromolecules to damage proteins, lipids, mitochondria, and DNA [16, 17]. The brain is especially susceptible to damage caused by oxidative stress because neurons contain low levels of endogenous antioxidant enzymes [12]. In neurodegenerative disorders, ROS mediates cellular apoptosis in damaged neurons, which might impair brain function [12, 15, 18].

In this study, i evaluated the effects of caffeinated and decaffeinated coffee on H<sub>2</sub>O<sub>2</sub>-induced neuronal apoptosis as well as signaling molecules involved, such as Bcl-2, Bcl-XL, caspase-3, and poly (ADP-ribose) polymerase (PARP). Our results indicate that both caffeinated and decaffeinated coffee similarly protect neurons from the effects of ROS and that chlorogenic acid (5-O-caffeoylquinic acid), formed by the esterification of quinic acid with trans-cinnamic acid, is a potential active molecule contributing to the neuroprotective effect.

## II. Materials and Methods

### 2.1. Materials

H<sub>2</sub>O<sub>2</sub> was purchased from Junsei Chemical (Tokyo, Japan). Poly-D-lysine (PDL), chlorogenic acid, and 4,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Thiazolyl blue tetrazolium bromide (MTT) was purchased from USB (Cleveland, OH, USA). Mg<sup>2+</sup>- and Ca<sup>2+</sup>-free Hanks' balanced salt solution (HBSS), neurobasal medium, B27, L-glutamine (200 mM), and penicillin/streptomycin were obtained from Gibco BRL (Carlsbad, CA, USA). Trypsin was obtained from In Vitrogen (Carlsbad, CA, USA). Anti-Bcl-2, anti-pro-PARP antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-xL, and anti-caspase-3 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-NQO1 antibody was obtained from Abcam (Cambridge, United Kingdom) and anti-β-actin antibody was purchased from Sigma–

Aldrich. All other chemicals used were of analytic grade and were purchased from Sigma–Aldrich.

## **2.2. Primary neuronal culture**

Cerebral cortices from littermate embryos (gestation day 14) were removed, placed in ice-cold HBSS, centrifuged (300 x g, 2 min), and digested in 0.05% trypsin in HBSS at 37 C for 10 min. Tissues were washed twice with HBSS and resuspended in Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cell suspension was sieved through a cell strainer (70 µm; BD Biosciences, San Jose, CA, USA) and plated on PDL-coated plates. After 45 min of initial plating, the medium was changed to new Neurobasal medium supplemented with 2% B27, 2 mM L-glutamine, and 1% penicillin/streptomycin. Cultures were maintained at 37 C in a humidified atmosphere of 5% CO<sub>2</sub>.

### **2.3. Caffeinated and decaffeinated coffee sample preparation**

Distilled water (80 °C, 100 ml) was added to 10 g of commercially prepared instant coffee (Maxim Original, Dongsuh Food, Seoul, Korea) and instant decaffeinated coffee (Maxim Decaffeinated, Dongsuh Food) and stirred for 5 min. The solution was then filtered through a membrane filter under a vacuum. A stock solution of 100 mg/ml caffeinated and decaffeinated coffee was used in this study.

### **2.4. MTT assay**

MTT is metabolized to an insoluble purple formazan by mitochondrial dehydrogenases, which are active only in live cells. Cell viability was measured based on the formation of a purple formazan metabolite, which was solubilized by the addition of dimethyl sulfoxide (DMSO). Primary neuronal cultures ( $2 \times 10^5$  cells/well in 96-well plates) were incubated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h with or without pretreatment with chlorogenic acid, and then treated with 1 mg/ml MTT for 2 h. The

dark blue formazan crystals in the intact neurons were dissolved in DMSO, and the absorbance at 540 nm was measured with a microplate reader. The results are expressed as the percent in absorbance relative to that in the control neurons.

## **2.5. DAPI staining**

The fluorescent dye DAPI was used to detect nuclear fragmentation, which is a characteristic of apoptotic cells. Primary neuronal cultures ( $5 \times 10^5$  cells/well in 24-well plates) were incubated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h with or without pretreatment with caffeinated coffee, decaffeinated coffee, or chlorogenic acid, and then washed with phosphate-buffered saline (PBS) and fixed with 70% ethanol for 20 min. The fixed cells were then washed with PBS and stained with 3  $\mu$ g/ml DAPI. Following 10 min of incubation, the cells were again washed with PBS, and the plates were observed under a fluorescence microscope Olympus I x 51 (Olympus Optical, Tokyo, Japan).

## 2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Primary neuronal cultures ( $4 \times 10^6$  cells in a 6 cm dish) were treated with caffeinated coffee, decaffeinated coffee, or chlorogenic acid for 24 h and harvested in RNAiso Plus (Takara Bio, Inc., Shiga, Japan). After RT with oligo-dT primers using a PrimeScript™ 1<sup>st</sup> strand cDNA Synthesis Kit (Takara Bio, Inc.), the cDNA was probed using the following primers (Bioneer, Daejeon, Korea): NADPH:quinine oxidoreductase 1 (NQO1), 50-CATTCTGAAAGGCTGGTTTA-30, 50-CTAGCTTTGATCTGGTTGTC AG-30 , heme oxygenase-1 (HO-1), 50-TACACATCCAAGCCGAGAAT-30, 50-GTTCCTCTGTCAGCATCACC-30, glutamate-cysteine ligase catalytic subunit (GCLC), 50-ACAAGCACCCCGCTCGGT-30, 50-CTCCAGGCCTCTCTCCTCCC-30 , glutamate-cysteine ligase regulatory subunit (GCLM), 50-ACCTGGCCTCCTGCTGTGTG-30, 50-GGTCGGTGAGCTGTGGGTGT-30,  $\beta$ -actin, 50-TGGTGGGTATGGG TCAGAAGGACTC-30, 50-CATGGCTGGGGTTG A AGGTCTCA-30 . The reaction products were

separated in an 1.5% continuous agarose gel.

## **2.7. Western blot analysis**

Primary neuronal cultures ( $4 \times 10^6$  cells in a 6-cm dish) were incubated with 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h with or without pretreatment with caffeinated coffee, decaffeinated coffee, or chlorogenic acid, washed, collected with ice-cold PBS, and centrifuged at 600 x g for 10 min. The cell pellets were resuspended in 100  $\mu\text{l}$  of ice-cold lysis buffer (Cell Signaling Technology) and incubated on ice for 30 min. After centrifugation at 1000 x g for 15 min, the supernatants were harvested and stored at  $-70^\circ\text{C}$ . The protein concentrations of lysates were determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Protein lysates were separated on a 12% discontinuous sodium dodecyl sulfate–polyacrylamide gel, and J. Kim et al. / *Neurochemistry International* 60 (2012) 466–474 467 transferred onto a polyvinylidene difluoride transfer membrane blocked with 5% skim milk containing 0.5 mM Tris–HCl (pH

7.5), 150 mM NaCl, and 0.05% Tween-20 for 2 h at room temperature. The membranes were subsequently incubated with the primary antibodies. After three washes with Tris-buffered saline with 0.1% Tween-20 (TBST), the blots were incubated with horseradish peroxidase-conjugated secondary antibodies in TBST with 5% skim milk at a 1:5000 dilution for 2 h at room temperature. The blots were then washed three times in TBST. The blots were developed using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, New York, NY, USA).

## **2.8. Western blot analysis**

All experiments were repeated at least three times unless otherwise stated. The results are presented as the mean  $\pm$  SE of triplicates. Comparisons between two groups were analyzed using Student's t-test. p-Values less than 0.05 were considered statistically significant.

### **III. Results**

#### **3.1. Caffeinated and decaffeinated coffee attenuate hydrogen peroxide induced neuronal cell death via apoptosis**

I first examined the neuroprotective effect of caffeinated and decaffeinated coffee against H<sub>2</sub>O<sub>2</sub>-induced cell death by observing the morphological change (Fig. 1A). Primary neuronal culture on days 5–6 showed the normal morphology of healthy neuron (a). However, 24 h treatment with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> increased the number of neuron that had condensed cell bodies and cleaved neuritis (b). Cells pretreated with caffeinated or decaffeinated coffee at 50  $\mu$ g /ml for 1 h showed similar morphology to control neuron (c and d).

Nuclear condensation is a morphological characteristic of apoptosis [19]. Neuronal cell death via apoptosis was determined by the nuclear condensation visualized by DAPI staining (Fig. 1B). Treatment with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h resulted in the condensation of nuclei in

primary neuronal cells (b, arrows). Pretreatment with 50 µg/ml caffeinated or decaffeinated coffee for 1 h dramatically inhibited H<sub>2</sub>O<sub>2</sub>-induced nuclear condensation (c and d). The number of condensed nuclei per total nuclei of at least six random fields per sample was counted and the relative percentage of control was described in Fig. 1C. The data indicated that both caffeinated and decaffeinated coffee similarly inhibit H<sub>2</sub>O<sub>2</sub>-induced apoptosis in primary neuronal culture.

### **3.2 Caffeinated and decaffeinated coffee prevent hydrogen peroxide induced downregulation of Bcl-2, Bcl-XL, and cleavage of caspase-3 and pro-PARP**

The Bcl family inhibits the activation of the caspase-3 cascade and apoptosis [20]. Bcl-2 and Bcl-XL promote cell survival and down-regulation of Bcl-2 and Bcl-XL occurs during apoptosis [21, 22]. In our study, treatment with 50 µM H<sub>2</sub>O<sub>2</sub> for 24 h in primary neuronal cultures decreased the anti-apoptotic protein level of Bcl-2 and Bcl-XL, but the

effects of H<sub>2</sub>O<sub>2</sub> were markedly attenuated by pretreatment with caffeinated or decaffeinated coffee at 50 µg/ml for 1 h (Fig. 2A and B).

I also investigated whether the increased expression of Bcl-2 and Bcl-XL in response to caffeinated and decaffeinated coffee leads to a lack of caspase-3 activation. Caspase-3 is activated through proteolytic processing from its inactive zymogen into activated fragments [23]. Treatment with 50 µM H<sub>2</sub>O<sub>2</sub> for 24 h in primary neuronal culture increased the level of cleaved-caspase-3 (Fig. 2A). Preincubation with 50 µg/ml of caffeinated or decaffeinated coffee for 1 h decreased the level of cleaved-caspase-3 in neurons (Fig. 2A).

Impaired mitochondrial membrane potential and caspase activation leads to the cleavage of PARP from its full-length form (116 kDa) to its cleaved form (89 kDa) [24]. The exposure of primary neuronal culture to 50 µM H<sub>2</sub>O<sub>2</sub> for 24 h induced PARP cleavage and decreased the level of full-length pro-PARP; however, the effect was inhibited by pretreatment with 50 µg/ml of caffeinated or decaffeinated coffee for 1 h

(Fig. 2A and C). These results suggest that caffeinated and decaffeinated coffee significantly attenuate H<sub>2</sub>O<sub>2</sub>-induced apoptotic neuronal cell death by blocking the H<sub>2</sub>O<sub>2</sub>-associated down-regulation of Bcl-2 and Bcl-XL as well as cleavage of caspase-3 and pro-PARP.

### **3.3 Chlorogenic acid inhibits hydrogen peroxide induced neuronal cell death via apoptosis**

Recently, increasing attention has been directed to chlorogenic acid because of its abundance in caffeinated and decaffeinated coffee [7, 9]. To investigate the neuroprotective effect of chlorogenic acid, primary neuronal cultures were treated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h with or without preincubation of 12.5–100  $\mu$ M chlorogenic acid for 1 h (Fig. 3A). Neuronal cells exposed to 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h exhibited significant cytotoxicity compared to control cells (Fig. 3A). Preincubation of 12.5–100  $\mu$ M chlorogenic acid for 1 h significantly protected H<sub>2</sub>O<sub>2</sub>-mediated neuronal cell death (Fig. 3A). I observed that chlorogenic acid clearly

protected neurons from H<sub>2</sub>O<sub>2</sub> and that the cell bodies and neurites of neuronal cells treated with chlorogenic acid were much healthier than those treated with H<sub>2</sub>O<sub>2</sub> alone (Fig. 3B, b and c).

Treatment with 50 μM H<sub>2</sub>O<sub>2</sub> for 24 h in primary neuronal culture resulted in the condensation of nuclei in neuronal cells, as determined by DAPI staining; however, this was significantly decreased by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 3C, arrows). The number of condensed nuclei per total nuclei of at least six random fields per sample was counted and the relative percentage of control was described in Fig. 3D. The data indicated that chlorogenic acid significantly inhibits H<sub>2</sub>O<sub>2</sub>-induced increase in the number of condensed nuclei and apoptosis in primary neuronal culture.

### **3.4 Chlorogenic acid prevents hydrogen peroxide induced down-regulation of Bcl-2, Bcl-XL, and cleavage of caspase-3 and pro-PARP**

H<sub>2</sub>O<sub>2</sub> decreased the protein level of Bcl-2 and Bcl-XL, but the effects were markedly attenuated by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 4A–C). Western blot analysis revealed that cleavage of caspase-3 induced by treatment with 50 μM H<sub>2</sub>O<sub>2</sub> for 24 h was inhibited by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 4A and D). The exposure of neuronal cells to 50 μM H<sub>2</sub>O<sub>2</sub> for 24 h induced pro-PARP cleavage; however, the effect was inhibited by pretreatment with 50 μM chlorogenic acid for 1 h (Fig. 4A and E). These results suggest that chlorogenic acid attenuates the H<sub>2</sub>O<sub>2</sub>-induced apoptosis of neuronal cells by blocking the down-regulation of Bcl-2 and Bcl-XL as well as cleavage of caspase-3 and pro-PARP.

### **3.5 Caffeinated coffee, decaffeinated coffee, and chlorogenic acid upregulate the expression of NQO1**

It has been reported that coffee compounds can activate the neuroprotective transcription factor NF-E2-related factor 2 (Nrf2) [6]. I hypothesized that the protective effects of caffeinated coffee, decaffeinated coffee, and chlorogenic acid against H<sub>2</sub>O<sub>2</sub>-induced neuron cell death may be mediated through the Nrf2-mediated antioxidant enzyme NQO1. I determined whether the level of expression of a known Nrf2-dependent gene, NQO1, in primary neuronal culture is altered by treatment with 50 µg/ml caffeinated or decaffeinated coffee for 24 h. Total RNA was isolated from primary neuronal cultures and cDNA was synthesized for PCR amplification of NQO1. The mRNA expression of NQO1 was not altered by the treatment of 50 µM H<sub>2</sub>O<sub>2</sub> for 24 h, however, significantly increased in primary neuronal cultures pretreated together with caffeinated or decaffeinated coffee for 1 h (Fig. 5A and B). Chlorogenic acid, the abundant polyphenolic phytochemical in caffeinated and decaffeinated coffee, also dramatically increased NQO1 mRNA expression in primary neuronal culture when cells were pretreated with 50 µM chlorogenic acid

for 1 h together with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h (Fig. 5C and D).

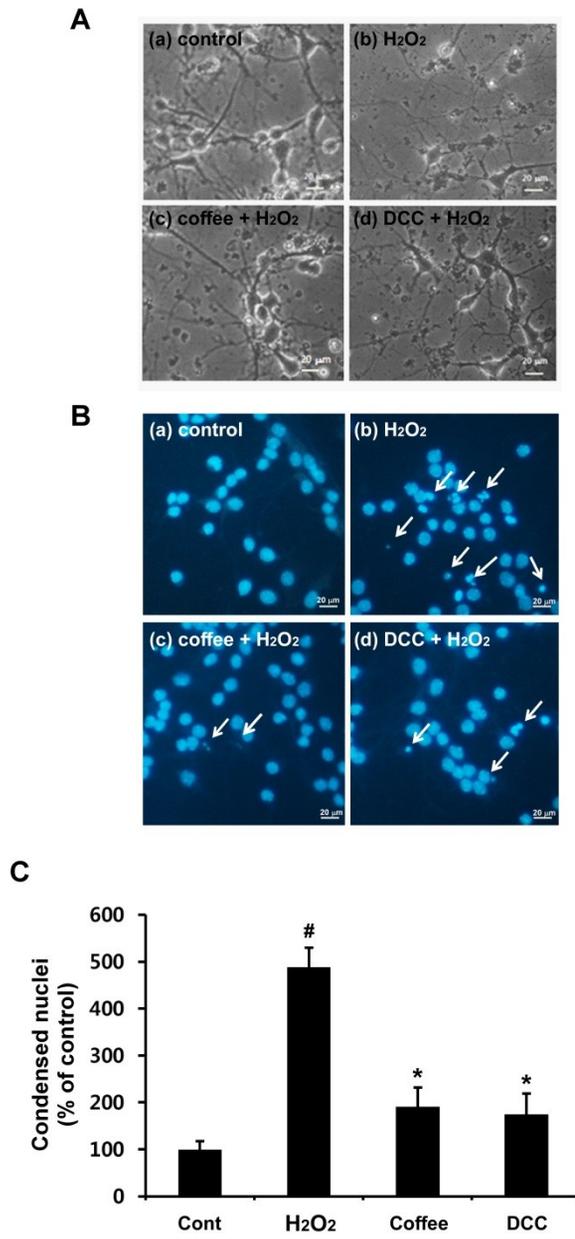
I also observed the protein expression of NQO1 altered by H<sub>2</sub>O<sub>2</sub> and NQO1 in primary neuronal culture. The protein expression of NQO1 was not altered by the treatment of 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h, however, significantly increased in primary neuronal cultures pretreated together with caffeinated or decaffeinated coffee as well as chlorogenic acid for 1 h (Fig. 5E–H).

### **3.6 Caffeinated coffee, decaffeinated coffee, and chlorogenic acid do not alter the expression of HO-1, GCLC, and GCLM**

I determined whether the level of expression of antioxidant enzyme genes, HO-1, GCLC and GCLM, in primary neuronal culture is altered by treatment with 50  $\mu$ g/ml caffeinated or decaffeinated coffee. Total RNA was isolated from primary neuronal cultures and cDNA was synthesized for PCR amplification of HO-1, GCLC and GCLM. The mRNA expression of HO-1, GCLC and GCLM was not altered by the

treatment of 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h, as well as, was not changed in primary neuronal cultures pretreated together with caffeinated or decaffeinated coffee for 1 h (Fig. 6A and B). Chlorogenic acid also did not alter the mRNA expression of HO-1, GCLC and GCLM in primary neuronal culture when cells were pretreated with 50  $\mu\text{M}$  chlorogenic acid for 1 h together with 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h (Fig. 6C and D).

**Fig. 1**

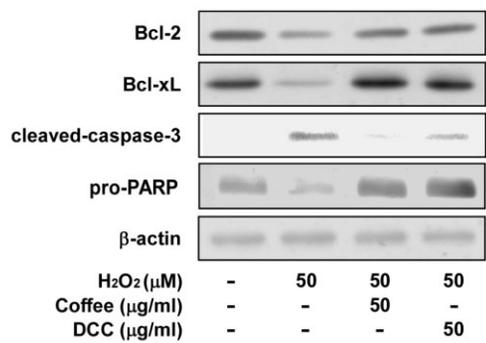


**Figure 1. Neuroprotective effects of caffeinated and decaffeinated coffee (DCC) on hydrogen peroxide induced neuronal cell death.**

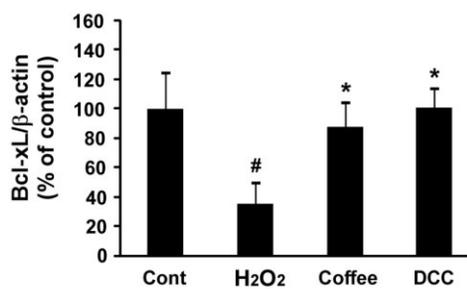
Cells were pretreated with caffeinated or decaffeinated coffee (DCC) at 50  $\mu\text{g/ml}$  for 1 h followed by 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. (A) The viability of neuronal cells was determined by cell morphology visualized under a microscope, and the phase contrast images were presented. (B) The apoptotic neuronal cells were examined by DAPI staining under a fluorescence microscope. The arrows indicate the condensed nuclei of apoptotic neuronal cells. (C) The number of condensed nuclei per total nuclei of at least six random fields per sample was counted. Relative levels of condensed nuclei, expressed as a percentage of control values, are presented as means  $\pm$  SE (n = 3). # Indicates significant difference at  $p < 0.05$  comparing control vs.  $\text{H}_2\text{O}_2$ , and \*,  $\text{H}_2\text{O}_2$  vs. coffee or DCC.

**Fig. 2**

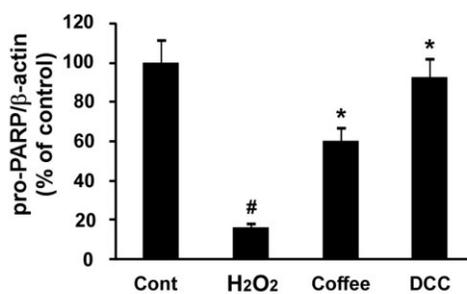
**A**



**B**



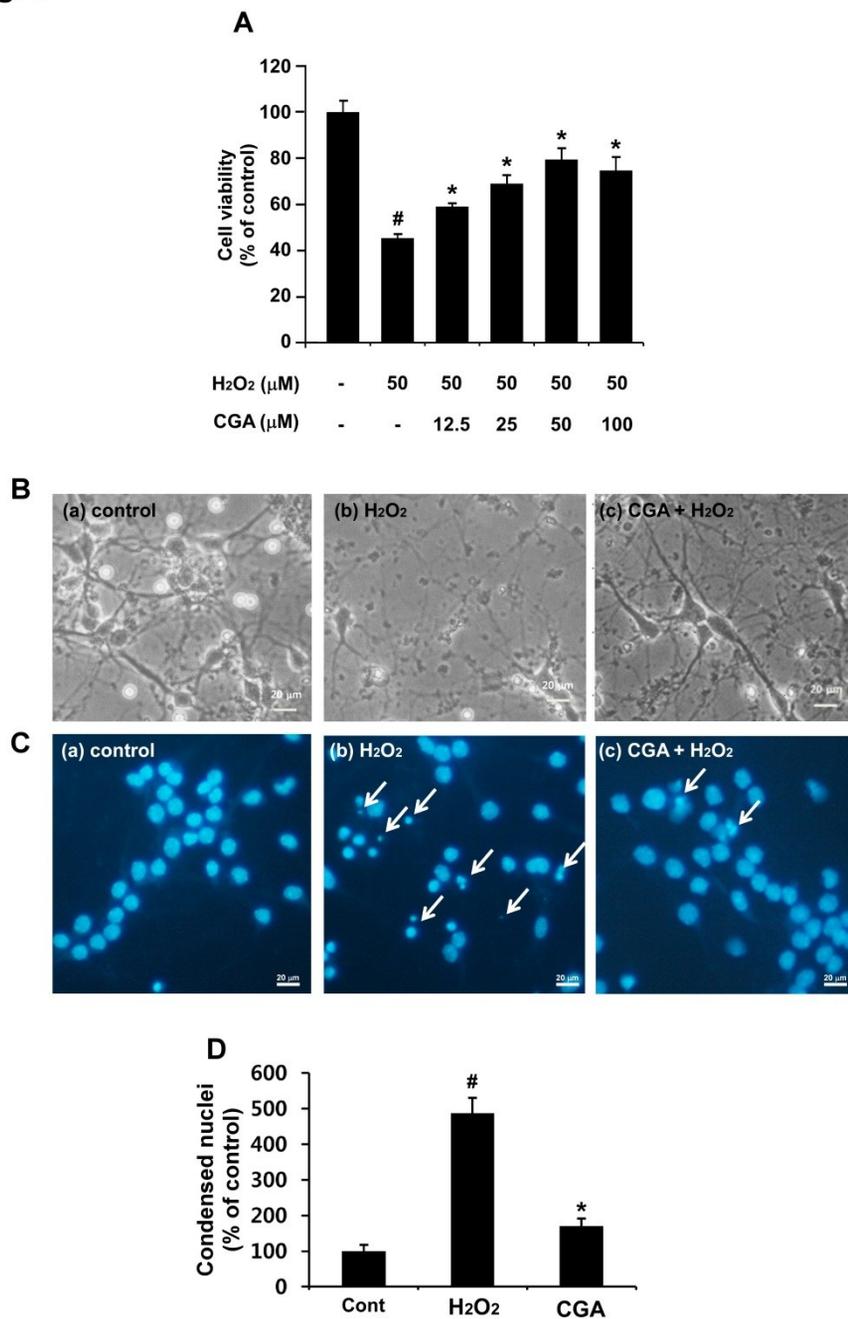
**C**



**Figure 2. Anti-apoptotic effects of caffeinated and decaffeinated coffee (DCC) on hydrogen peroxide induced down-regulation of Bcl-2 and Bcl-xL as well as cleavage of caspase-3 and pro-PARP in primary neuronal culture.**

Cells were pretreated with caffeinated or decaffeinated coffee (DCC) at 50  $\mu\text{g/ml}$  for 1 h followed by 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. (A) The levels of Bcl-2, Bcl-xL, and cleaved caspase-3 and pro-PARP were examined by Western blot analysis.  $\beta$ -actin was measured to confirm uniform protein loading. The ratio of (B) Bcl-xL/ $\beta$ -actin and (C) pro-PARP/ $\beta$ -actin was determined by densitometry. Data are presented as means  $\pm$  SE of three independent experiments. # Indicates significant difference at  $p < 0.05$  comparing control vs.  $\text{H}_2\text{O}_2$ , and \*,  $\text{H}_2\text{O}_2$  vs. coffee or DCC.

**Fig. 3**

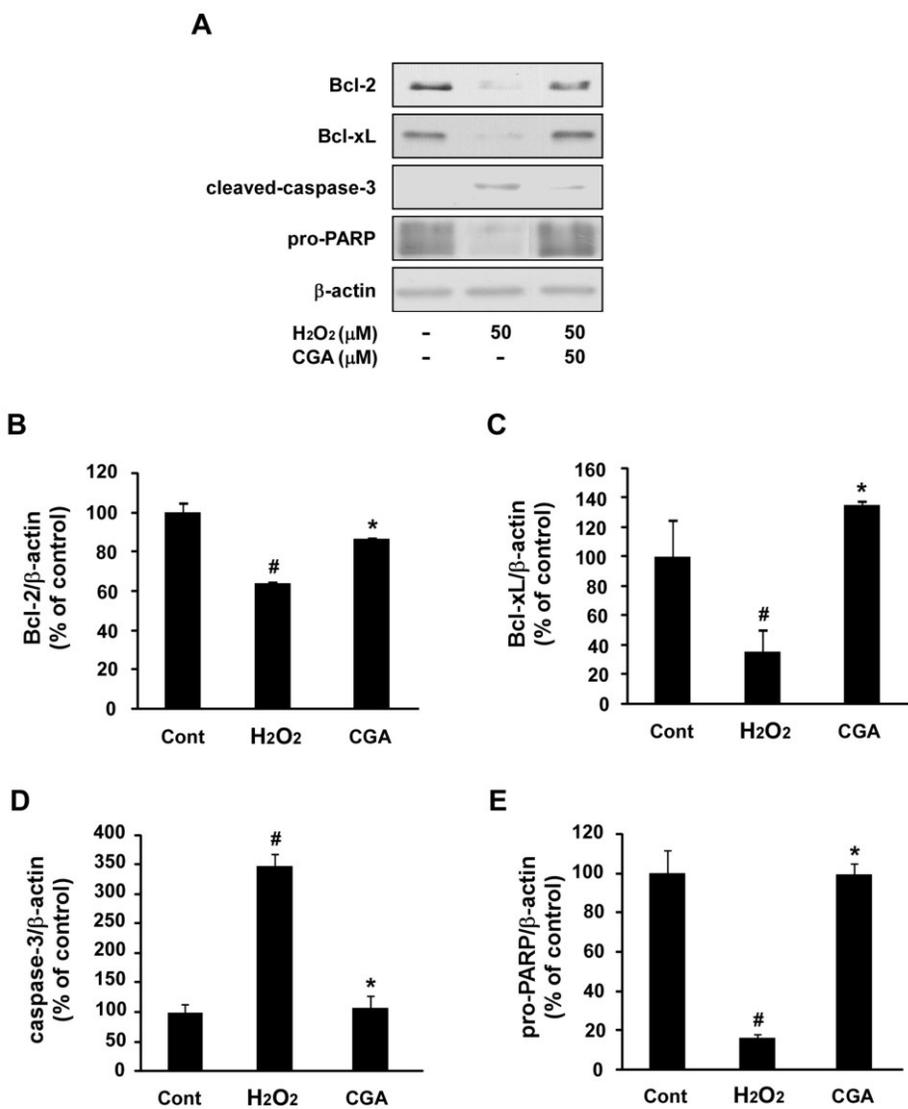


**Figure 3. Neuroprotective effect of chlorogenic acid (CGA) on hydrogen peroxide induced neuronal cell death. (A) Cells were pretreated with chlorogenic acid (CGA) at 12.5–100  $\mu$ M for 1 h followed by 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h.**

The viability of the neuronal cells was measured by MTT assay. The relative cell viabilities, expressed as a percentage of control values, are presented as means  $\pm$  SE (n = 3). (B–D) Cells were pretreated with chlorogenic acid (CGA) at 50  $\mu$ M for 1 h followed by 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h. (B) The viability of the neuronal cells was visualized under a microscope, and the phase contrast images were presented. (C) The apoptotic neuronal cells were examined by DAPI staining under a fluorescence microscope. The arrows indicate the condensed nuclei of apoptotic neuron. (D) The number of condensed nuclei per total nuclei of at least six random fields per sample was counted. Relative levels of condensed nuclei, expressed as a percentage of control values, are presented as means  $\pm$  SE (n = 3). # Indicates significant difference at p <

0.05 comparing control vs. H<sub>2</sub>O<sub>2</sub>, and \*, H<sub>2</sub>O<sub>2</sub> vs. CGA.

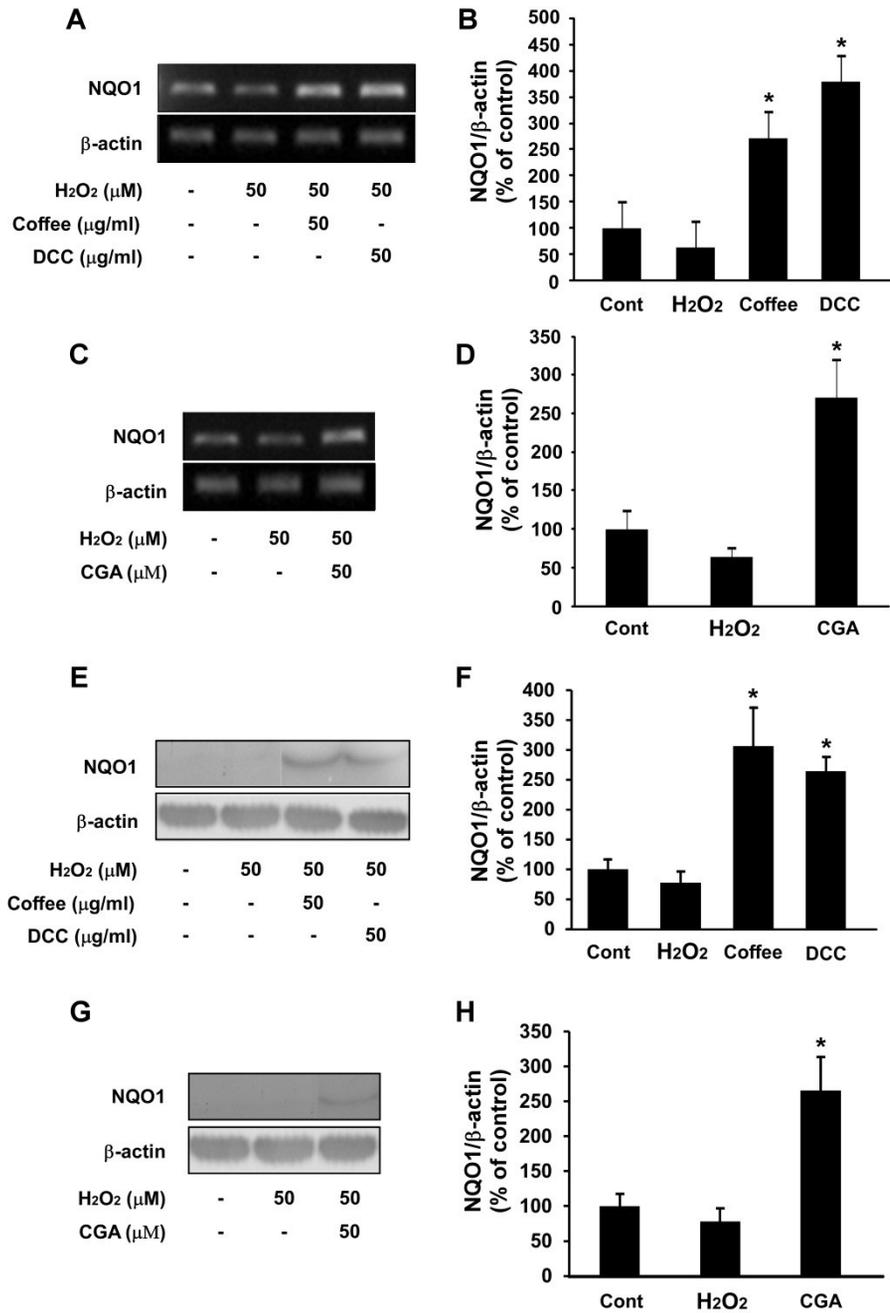
Fig. 4



**Figure 4. Anti-apoptotic effects of chlorogenic acid (CGA) on hydrogen peroxide induced down-regulation of Bcl-2 and Bcl-xL as well as cleavage of caspase-3 and pro-PARP in primary neuronal culture.**

Cells were pretreated with chlorogenic acid (CGA) at 50  $\mu$ M for 1 h followed by 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 24 h. (A) The levels of Bcl-2, Bcl-xL, cleaved caspase-3, and cleaved pro-PARP were examined by Western blot analysis.  $\beta$ -actin was measured to confirm uniform protein loading. The ratio of (B) Bcl-2/ $\beta$ -actin, (C) Bcl-xL/ $\beta$ -actin, (D) caspase-3/ $\beta$ -actin, and (E) pro-PARP/ $\beta$ -actin was determined by densitometry. Data are presented as means  $\pm$  SE of three independent experiments. # Indicates significant difference at  $p < 0.05$  comparing control vs. H<sub>2</sub>O<sub>2</sub>, and \*, H<sub>2</sub>O<sub>2</sub> vs. CGA.

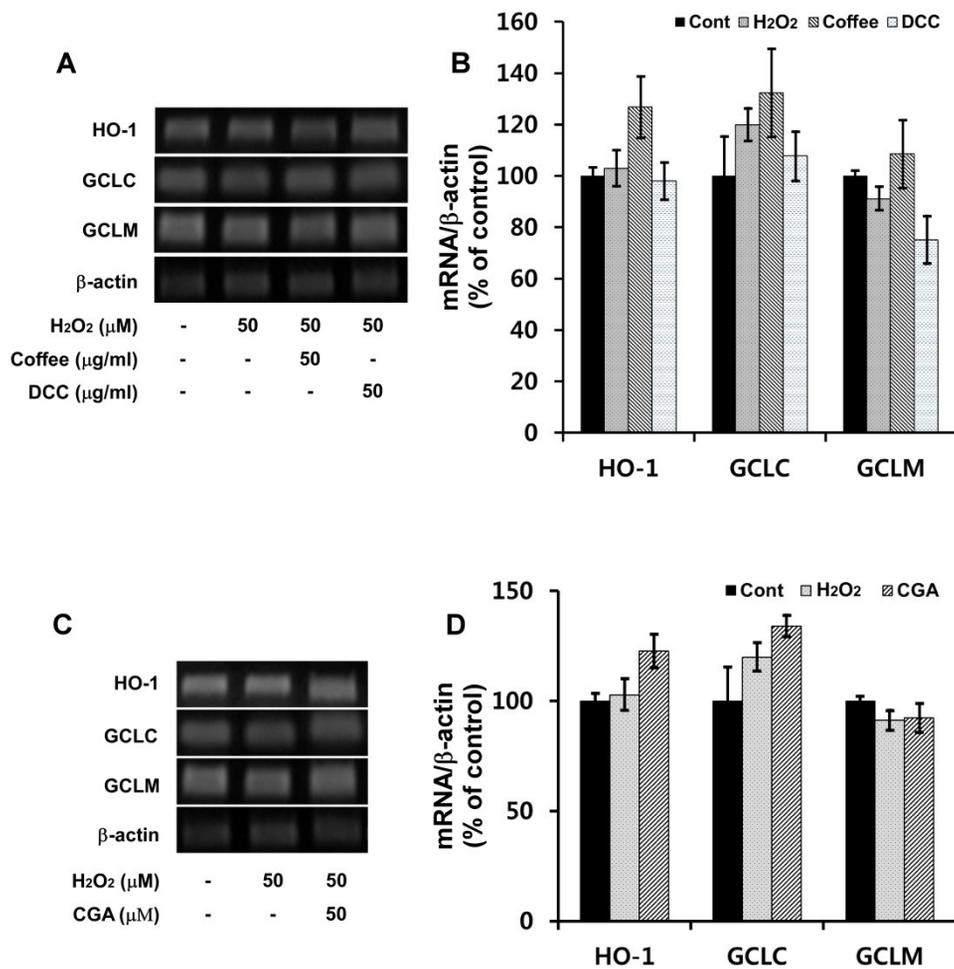
**Fig. 5**



**Figure 5. Caffeinated coffee-, decaffeinated coffee- (DCC-), and chlorogenic acid (CGA)-mediated NQO1 expression in primary neuronal culture.**

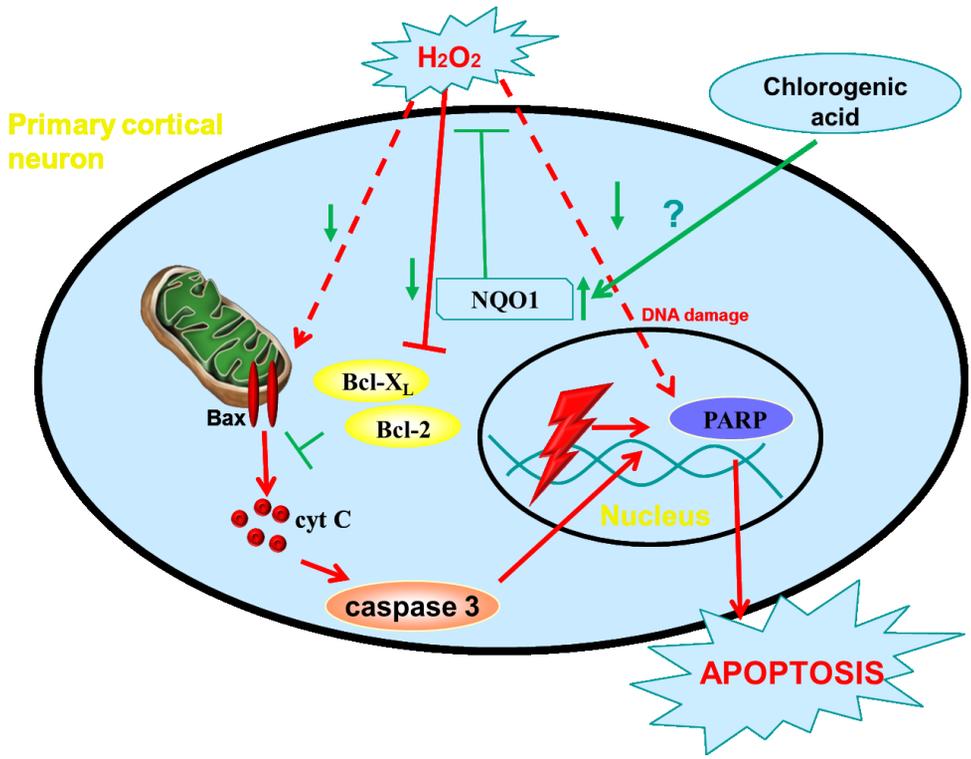
Cells were pretreated with caffeinated coffee and decaffeinated coffee (DCC) at 50  $\mu\text{g/ml}$ , and chlorogenic acid (CGA) at 50  $\mu\text{M}$  for 1 h followed by 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. (A and C) The mRNA levels of NQO1 were examined by RT-PCR.  $\beta$ -actin was measured to confirm uniform loading. (B and D) The ratio of NQO-1/ $\beta$ -actin was determined by densitometry. (E and G) The protein levels of NQO1 were examined by Western blot analysis.  $\beta$ -actin was measured to confirm uniform loading. (F and H) The ratio of NQO-1/ $\beta$ -actin was determined by densitometry. Data are presented as means  $\pm$  SE of three independent experiments. \* indicates significant difference at  $p < 0.05$  comparing  $\text{H}_2\text{O}_2$  vs. CGA.

**Fig. 6**



**Figure 6. Caffeinated coffee-, decaffeinated coffee- (DCC-), and chlorogenic acid (CGA)-mediated HO-1, GCLC and GCLM expression in primary neuronal culture.**

Cells were pretreated with caffeinated coffee and decaffeinated coffee (DCC) at 50  $\mu\text{g/ml}$ , and chlorogenic acid (CGA) at 50  $\mu\text{M}$  for 1 h followed by 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 24 h. (A and C) The mRNA levels of HO-1, GCLC and GCLM were examined by RT-PCR.  $\beta$ -actin was measured to confirm uniform loading. (B and D) The ratio of HO-1/ $\beta$ -actin, GCLC/ $\beta$ -actin and GCLM/ $\beta$ -actin was determined by densitometry. Data are presented as means  $\pm$  SE of three independent experiments.



## IV. Discussion

Our work indicates that both caffeinated and decaffeinated coffee similarly protect neuron against H<sub>2</sub>O<sub>2</sub>-mediated apoptosis. Chlorogenic acid, a major phenolic phytochemical found in both caffeinated and decaffeinated coffee, also conferred protection against H<sub>2</sub>O<sub>2</sub>-induced apoptotic neuronal loss. Our study demonstrated that caffeinated coffee, decaffeinated coffee, and chlorogenic acid up-regulated the anti-apoptotic proteins Bcl-2 and Bcl-XL and inhibited pro-apoptotic cleavage of caspase-3 and pro-PARP, which probably exerted a neuroprotective effect on this population of cells.

Here, we also found that caffeinated coffee, decaffeinated coffee, and chlorogenic acid increased the expression of NQO1. NQO1, previously known as DT diaphorase, is a flavoprotein that catalyzes the two-electron reduction and detoxification of quinones and their derivatives [25, 26]. NQO1 is a component for the plasma membrane redox system,

which provides electrons for energy metabolism and recycling of antioxidants [27]. NQO1 maintains cellular levels of ubiquinol and vitamin E, two important biological antioxidants involved in the detoxification of ROS [28, 29]. Moreover, NQO1 is expressed in neural cells and up-regulated in response to mitochondrial impairment and protect the cells against oxidative stress [27, 30-32].

Overexpression of NQO1 was found to protect neurons against amyloid- $\beta$  cytotoxicity [27]. Cross-species microarray analysis suggested that NQO1 mediates neuroprotective pathways in aging and Alzheimer's disease [33]. NQO1 also plays a critical role in protecting neuronal cells against dopamine or 6-hydroxydopamine-induced oxidative stress [34, 35]. Indeed, a recent observation suggested that polymorphism of NQO1 is associated with an increased risk of developing Parkinson's disease [36]. It has been reported that coffee compounds can activate neuroprotective transcription factor Nrf2, which regulates the expression of antioxidant enzyme NQO1 [6]. The possible neuroprotection mediated by caffeinated

coffee, decaffeinated coffee, and chlorogenic acid against H<sub>2</sub>O<sub>2</sub> may be at least partly due to Nrf2-induced modulatory effects on NQO1 expression.

Bcl-2 and Bcl-XL promote cell survival and down-regulation of Bcl-2 and Bcl-XL occurs during apoptosis [21, 22]. The Bcl family of proteins inhibits the activation of the caspase-3 cascade and apoptosis [20]. Impaired mitochondrial membrane potential and caspase activation lead to the cleavage of PARP from its full-length form (116 kDa) to its cleaved form (89 kDa) [24, 37]. It has been reported that dicoumarol, a potent inhibitor of NQO1, decreases the protein level of Bcl-xL and potentiates to induce cytotoxicity [38]. On the other hand, ladostigil, a drug which elevates the expression of NQO1, was shown to induce the level of Bcl-2 gene and protein [39]. These evidences suggest that coffees and chlorogenic acid-mediated increase in NQO1 expression induce the expression of anti-apoptotic protein Bcl-xL and Bcl-2, activate caspase-3, and lead to the cleavage of PARP.

Concentrations of coffee bioactives in the brain are important determinants of whether the protective effect observed is biologically relevant. So far, there is no direct report as to whether chlorogenic acids can pass through the blood–brain barrier. However, it has been found that chlorogenic acid is neuroprotective against scopolamine-induced amnesia and significantly improved the impairment of short-term or working memory induced by scopolamine [40]. Chlorogenic acid also affected spontaneous locomotor activity in a mouse model and improved clinical rating scores in rabbits following multiple infarct ischemic strokes, suggesting that chlorogenic acid or its metabolites could pass the blood–brain barrier and exert their effort [41, 42]. Given the high plasma level after coffee ingestion and the small molecular weight (chlorogenic acid, MW 354), chlorogenic acid is likely to permeate the blood–brain barrier [43].

The amount of chlorogenic acid in a 200 ml cup of coffee is about 70–350 mg [8, 44]. The pharmacokinetic data of chlorogenic acid after

consumption of caffeinated or decaffeinated coffee is not yet enough to determine, however, one study showed that human plasma concentration of chlorogenic acid reached about  $3.14 \pm 1.64 \mu\text{M}$  after oral ingestion of 190 ml of coffee drink containing  $1068 \pm 49 \mu\text{mol}$  (67.26 mg) chlorogenic acid [45]. Studies on bioavailability after several months and years of chlorogenic acid-rich coffee consumption are required to fully understand the neuroprotective effects. On the other hand, ingredients other than chlorogenic acid present in caffeinated and decaffeinated coffee may also be involved in neuroprotection. For example, chlorogenic acid lactones contributed to the increased protective effects of coffee against neuronal cell death [43]. Caffeic acid, present in coffee, has been shown to be neuron-protective in vivo under pathological conditions [46]. Kahweol and cafestol were also suggested as antioxidative and neuroprotective components in coffee [6, 47]. Combination of these phytochemicals together with chlorogenic acid may synergistically promote the neuro-protective effects of caffeinated and decaffeinated coffee.

In summary, the results of this study show that caffeinated and decaffeinated coffee inhibited H<sub>2</sub>O<sub>2</sub>-induced apoptotic neuronal death and that chlorogenic acid might be largely responsible for these effects. This protection occurred through the inhibition of H<sub>2</sub>O<sub>2</sub>-induced down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-XL as well as the blockage of H<sub>2</sub>O<sub>2</sub>-induced pro-apoptotic cleavage of caspase-3 and pro-PARP in primary cortical neurons. I also found that caffeinated coffee, decaffeinated coffee, and chlorogenic acid induced the expression of NQO1, suggesting that these substances protect neurons by up-regulation of the antioxidant enzyme NQO1.

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## VI. 초록

신경퇴행성 질환은 과산화수소수를 포함한 여러 활성산소에 의한 산화적 스트레스와 아주 밀접한 관련이 되어있다. 역학조사에서 커피는 신경을 보호하는 효능이 있음이 알려져 왔으나, 그 구체적인 작용기작에 대해서는 명확히 알려지지 않았다. 따라서 본 연구에서는 카페인이 함유된 커피, 카페인을 제거한 커피 및 두 커피에 모두 포함되어 있는 페놀성 파이토케미칼인 클로로젠산을 가지고 신경세포의 사멸억제효능 및 그 작용기전을 규명하였다. 먼저 본 연구에서는 과산화수소수로 유도한 세포사멸이 발생하는 것을 세포의 형태학적 분석 및 핵의 응축현상을 통해 확인하였으며, 카페인이 함유된 커피, 카페인을 제거한 커피 및 클로로젠산을 각각 전처리한 군에서 세포사멸이 유의적으로 감소하는 것을 광학현미경 및 형광현미경을 통해 확인할 수 있었다. 또한 각각의 샘플이 세포사멸기작을 저해하는 Bcl-2, Bcl-xL 등의 신호를 정상 수치로 유지시키고 세포사멸신호인 caspase-3의 활성을

저해하고 PARP의 활성을 줄이는 것을 확인함으로써, 커피 및 폴리페놀이 신경세포사멸을 억제하는 것을 분자생물학적 관점에서 증명하였다. 또한 본 연구에서는 커피 및 클로로겐산이 세포사멸억제효능을 보이는 원인이 항산화효소인 NQO1의 증가와 관련이 있음을 밝혔고, 이러한 NQO1의 증가가 과산화수소수로 유도되는 세포사멸기작으로부터 신경세포를 보호하는 것으로 추측하였다. 결론적으로, 카페인 함유 커피와 탈카페인 커피는 비슷한 신경보호효능을 보였고 이러한 원인은 두 커피에서 공통적으로 들어있는 클로로겐산에 의한 것임을 추측할 수 있었다.

주요어 : 카페인 함유 커피; 탈카페인 커피; 클로로겐산; NQO1;  
일차신경세포; 과산화수소수; 세포사멸