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Caffeinated coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid up-regulate NQO1 expression and prevent H_2O_2-induced apoptosis in primary cortical neurons

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ABSTRACT

Neurodegenerative disorders are strongly associated with oxidative stress, which is induced by reactive oxygen species including hydrogen peroxide (H_2O_2). Epidemiological studies have suggested that coffee may be neuroprotective, but the molecular mechanisms underlying this effect have not been clarified. In this study, we 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 oxidative neuronal death. H_2O_2-induced apoptotic nuclear condensation in neuronal cells was strongly inhibited by pretreatment with caffeinated coffee, decaffeinated coffee, or chlorogenic acid. Pretreatment with caffeinated coffee, decaffeinated coffee, or chlorogenic acid inhibited the H_2O_2-induced down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-X_L while blocking H_2O_2-induced pro-apoptotic cleavage of caspase-3 and pro-poly(ADP-ribose) polymerase. We 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_2O_2-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.

1. 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 (McCall et al., 1982). Several studies have reported that consumption of coffee or caffeine in both young and adult is associated with better cognitive performance (Hameleers et al., 2000; Jarvis, 1993). Other studies have suggested that coffee and caffeine may have protective effects against Alzheimer's disease and Parkinson's disease (Arendash and Cao, 2010; Ross et al., 2000b). 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 (Trinh et al., 2010).

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 (George et al., 2008). 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 (Clifford et al., 2007). Coffee contains approximately 7–9% polyphenolic chlorogenic acids but only 1% caffeine (George et al., 2008; Hoelzl et al., 2010; Ramalakshmi and Raghavan, 1999). Therefore, studying the effect of chlorogenic acids may be necessary to fully understand the neuropharmacological effects of caffeinated and decaffeinated coffee.

Abbreviations: H_2O_2, hydrogen peroxide; NQO1, NADPH:quinine oxidoreductase 1; ROS, reactive oxygen species; PARP, poly(ADP-ribose) polymerase.

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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 (Olanow, 1992, 1993; Richardson, 1993; Smith et al., 1991). Oxidative damage is mediated by reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion radical ($\cdot O_2^-$), and hydroxyl radical ($OH\cdot$) (Facchinetti et al., 1998; Olanow, 1993). 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 (Dizdaroglu et al., 1991; Vianello et al., 1990). The brain is especially susceptible to damage caused by oxidative stress because neurons contain low levels of endogenous antioxidant enzymes (Olanow, 1993). In neurodegenerative disorders, ROS mediates cellular apoptosis in damaged neurons, which might impair brain function (Barinaga, 1998; Facchinetti et al., 1998; Olanow, 1993).

In this study, we evaluated the effects of caffeinated and decaffeinated coffee on H_2O_2-induced neuronal apoptosis as well as signaling molecules involved, such as Bcl-2, Bcl-X_L, casepase-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.

2. Materials and methods

2.1. Chemicals and reagents

H_2O_2 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^{2+}- and Ca^{2+}-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 × *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_2.

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 (2×10^5 cells/well in 96-well plates) were incubated with 50 μM H_2O_2 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 (5×10^5 cells/well in 24-well plates) were incubated with 50 μM H_2O_2 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 μg/ml DAPI. Following 10 min of incubation, the cells were again washed with PBS, and the plates were observed under a fluorescence microscope Olympus l × 51 (Olympus Optical, Tokyo, Japan).

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

Primary neuronal cultures (4×10^6 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™ 1st strand cDNA Synthesis Kit (Takara Bio, Inc.), the cDNA was probed using the following primers (Bioneer, Daejeon, Korea): NADPH:quinine oxidoreductase 1 (NQO1), 5′-CATTCTGAAAGGCTGGTTTA-3′, 5′-CTAGCTTTGATCTGGTTGTCAG-3′, heme oxygenase-1 (HO-1), 5′-TACACATCCAAGCCGAGAAT-3′, 5′-GTTCCTCTGTCAG CATCACC-3′, glutamate-cysteine ligase catalytic subunit (GCLC), 5′-ACAAGCACC CCCGCTCGGT-3′, 5′-CTCCAGGCCTCTCTCCTCCC-3′, glutamate-cysteine ligase regulatory subunit (GCLM), 5′-ACCTGGCCTCCTGCTGT GTG-3′, 5′-GGTCGGTGAGCTGTGGGT GT-3′, β-actin, 5′-TGGTGGGTA TGGGTCAGAAGGACTC-3′, 5′-CATGGCTGGGGTTGA AGGTCTCA-3′. The reaction products were separated in an 1.5% continuous agarose gel.

2.7. Western blot analysis

Primary neuronal cultures (4×10^6 cells in a 6-cm dish) were incubated with 50 μM H_2O_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 × *g* for 10 min. The cell pellets were resuspended in 100 μl of ice-cold lysis buffer (Cell Signaling Technology) and incubated on ice for 30 min. After centrifugation at 1000 × *g* for 15 min, the supernatants were harvested and stored at −70 °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

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. Statistical analysis

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

3. Results

3.1. Caffeinated and decaffeinated coffee attenuate H_2O_2-induced neuronal cell death via apoptosis

We first examined the neuroprotective effect of caffeinated and decaffeinated coffee against H_2O_2-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 μM H_2O_2 increased the number of neuron that had condensed cell bodies and cleaved neuritis (b). Cells pretreated with caffeinated or decaffeinated coffee at 50 μg/ml for 1 h showed similar morphology to control neuron (c and d).

Nuclear condensation is a morphological characteristic of apoptosis (Tone et al., 2007). Neuronal cell death via apoptosis was determined by the nuclear condensation visualized by DAPI staining (Fig. 1B). Treatment with 50 μM H_2O_2 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_2O_2-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_2O_2-induced apoptosis in primary neuronal culture.

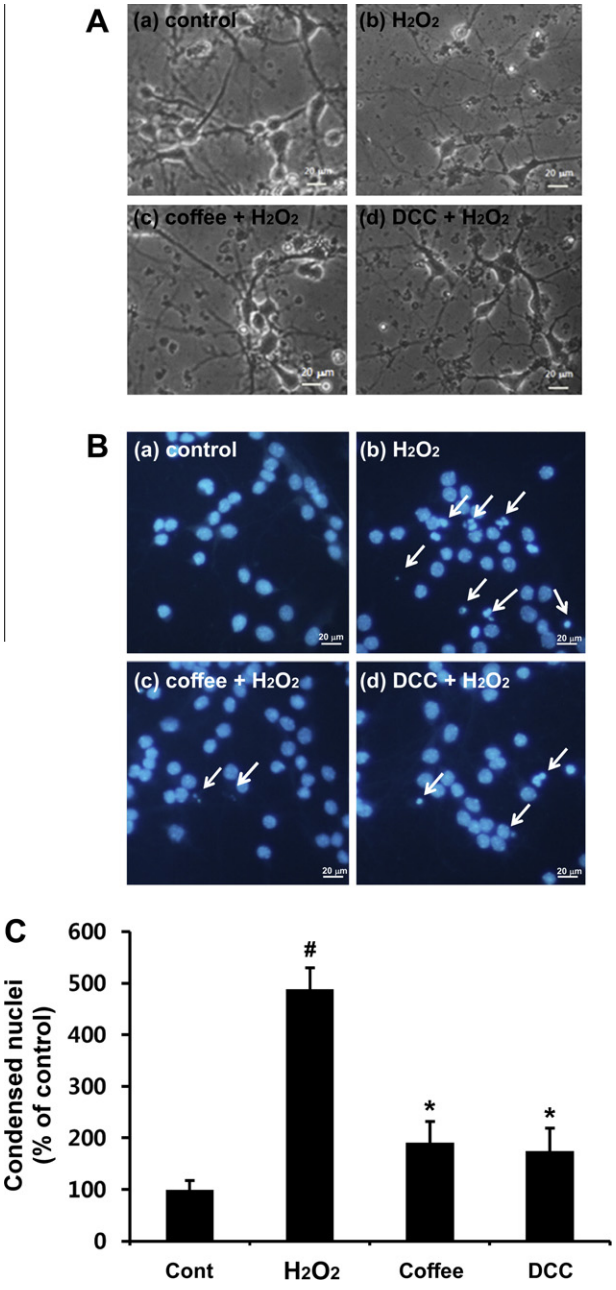

Fig. 1. Neuroprotective effects of caffeinated and decaffeinated coffee (DCC) on H_2O_2-induced neuronal cell death. Cells were pretreated with caffeinated or decaffeinated coffee (DCC) at 50 μg/ml for 1 h followed by 50 μM H_2O_2 for 24 h. (A) The viability of neuronal cells was determined by cell morphology 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 ± SE ($n = 3$). # Indicates significant difference at $p < 0.05$ comparing control vs. H_2O_2, and *, H_2O_2 vs. coffee or DCC.

3.2. Caffeinated and decaffeinated coffee prevent H_2O_2-induced down-regulation of Bcl-2, Bcl-$_X$L, and cleavage of caspase-3 and pro-PARP

The Bcl family inhibits the activation of the caspase-3 cascade and apoptosis (Zhao et al., 2003). Bcl-2 and Bcl-$_X$L promote cell survival and down-regulation of Bcl-2 and Bcl-$_X$L occurs during apoptosis (Olie et al., 2002; Tsujimoto et al., 1997). In our study, treatment with 50 μM H_2O_2 for 24 h in primary neuronal cultures decreased the anti-apoptotic protein level of Bcl-2 and Bcl-$_X$L, but the effects of H_2O_2 were markedly attenuated by pretreatment with caffeinated or decaffeinated coffee at 50 μg/ml for 1 h (Fig. 2A and B).

We also investigated whether the increased expression of Bcl-2 and Bcl-$_X$L 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 (Schindler et al., 2006). Treatment with 50 μM H_2O_2 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) (Brauns et al., 2005; Lee et al., 2006). The exposure of primary neuronal culture to 50 μM H_2O_2 for 24 h induced PARP cleavage and decreased the level of

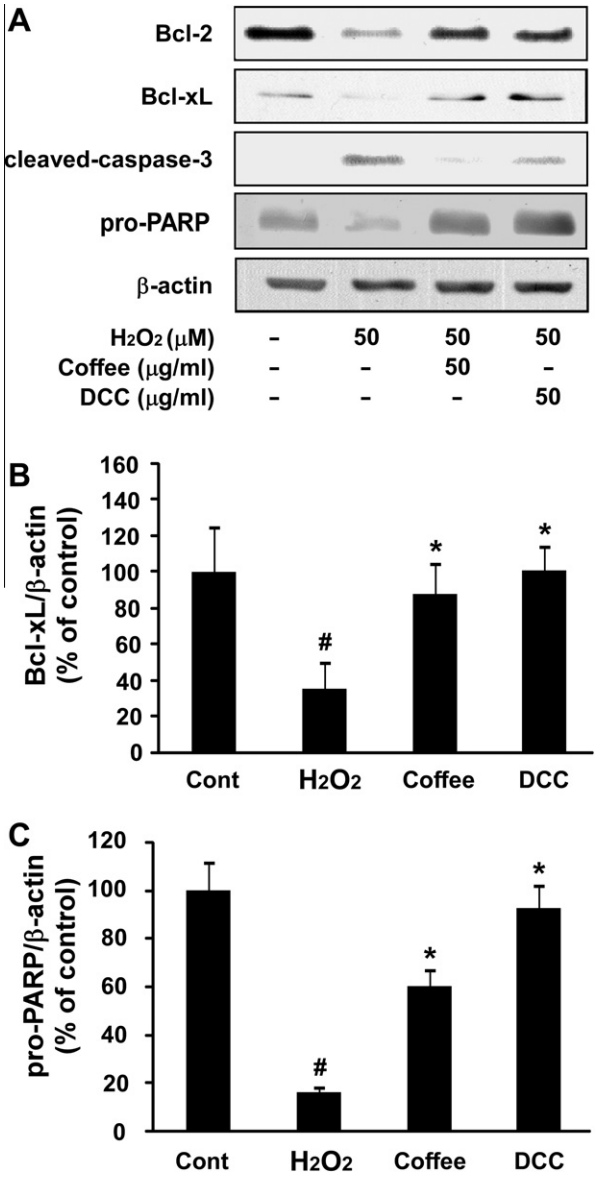

Fig. 2. Anti-apoptotic effects of caffeinated and decaffeinated coffee (DCC) on H_2O_2-induced down-regulation of Bcl-2 and Bcl-xL as well as cleavage of caspase-3 and pro-PARP in primary neuronal culture. Cells were pretreated with caffeinated or decaffeinated coffee (DCC) at 50 μg/ml for 1 h followed by 50 μM H_2O_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. β-Actin was measured to confirm uniform protein loading. The ratio of (B) Bcl-xL/β-actin and (C) pro-PARP/β-actin was determined by densitometry. Data are presented as means ± SE of three independent experiments. # Indicates significant difference at $p < 0.05$ comparing control vs. H_2O_2, and *, H_2O_2 vs. coffee or DCC.

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_2O_2-induced apoptotic neuronal cell death by blocking the H_2O_2-associated down-regulation of Bcl-2 and Bcl-$_X$L as well as cleavage of caspase-3 and pro-PARP.

3.3. Chlorogenic acid inhibits H_2O_2-induced neuronal cell death via apoptosis

Recently, increasing attention has been directed to chlorogenic acid because of its abundance in caffeinated and decaffeinated coffee (George et al., 2008; Hoelzl et al., 2010). To investigate the neuroprotective effect of chlorogenic acid, primary neuronal cultures were treated with 50 μM H_2O_2 for 24 h with or without preincubation of 12.5–100 μM chlorogenic acid for 1 h (Fig. 3A). Neuronal cells exposed to 50 μM H_2O_2 for 24 h exhibited significant cytotoxicity compared to control cells (Fig. 3A). Preincubation of 12.5–100 μM chlorogenic acid for 1 h significantly protected H_2O_2-mediated neuronal cell death (Fig. 3A). We observed that chlorogenic acid clearly protected neurons from H_2O_2 and that the cell bodies and neurites of neuronal cells treated with chlorogenic acid were much healthier than those treated with H_2O_2 alone (Fig. 3B, b and c).

Treatment with 50 μM H_2O_2 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_2O_2-induced increase in the number of condensed nuclei and apoptosis in primary neuronal culture.

3.4. Chlorogenic acid prevents H_2O_2-induced down-regulation of Bcl-2, Bcl-$_X$L, and cleavage of caspase-3 and pro-PARP

H_2O_2 decreased the protein level of Bcl-2 and Bcl-$_X$L, 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_2O_2 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_2O_2 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_2O_2-induced apoptosis of neuronal cells by blocking the down-regulation of Bcl-2 and Bcl-$_X$L as well as cleavage of caspase-3 and pro-PARP.

3.5. Caffeinated coffee, decaffeinated coffee, and chlorogenic acid up-regulate the expression of NQO1

It has been reported that coffee compounds can activate the neuroprotective transcription factor NF-E2-related factor 2 (Nrf2) (Trinh et al., 2010). We hypothesized that the protective effects of caffeinated coffee, decaffeinated coffee, and chlorogenic acid against H_2O_2-induced neuron cell death may be mediated through the Nrf2-mediated antioxidant enzyme NQO1. We 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_2O_2 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 μM H_2O_2 for 24 h (Fig. 5C and D).

We also observed the protein expression of NQO1 altered by H_2O_2 and NQO1 in primary neuronal culture. The protein expression of NQO1 was not altered by the treatment of 50 μM H_2O_2 for 24 h, however, significantly increased in primary neuronal

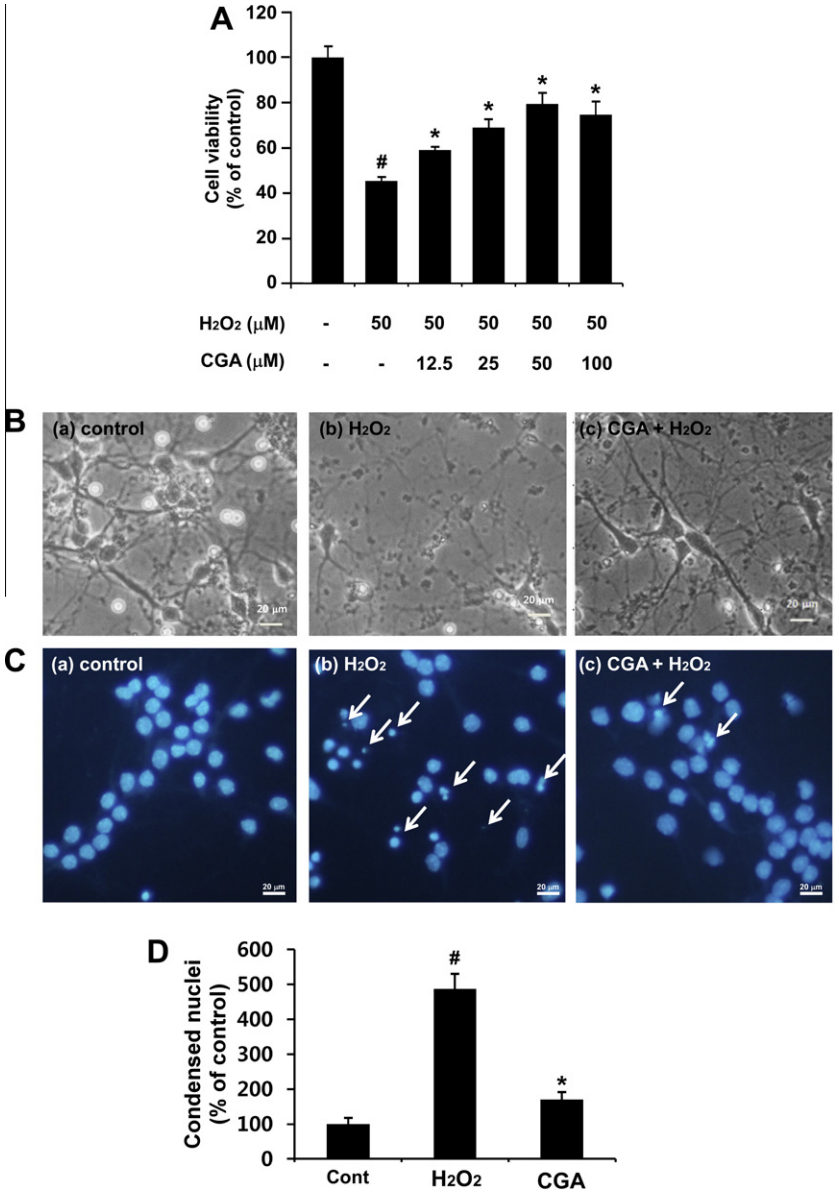

Fig. 3. Neuroprotective effect of chlorogenic acid (CGA) on H_2O_2-induced neuronal cell death. (A) Cells were pretreated with chlorogenic acid (CGA) at 12.5–100 μM for 1 h followed by 50 μM H_2O_2 for 24 h. The viability of the neuronal cells was measured by MTT assay. The relative cell viabilities, expressed as a percentage of control values, are presented as means ± SE ($n = 3$). (B–D) Cells were pretreated with chlorogenic acid (CGA) at 50 μM for 1 h followed by 50 μM H_2O_2 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 ± SE ($n = 3$). # Indicates significant difference at $p < 0.05$ comparing control vs. H_2O_2, and *, H_2O_2 vs. CGA.

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

We 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 μg/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 μM H_2O_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 μM chlorogenic acid for 1 h together with 50 μM H_2O_2 for 24 h (Fig. 6C and D).

4. Discussion

Our work indicates that both caffeinated and decaffeinated coffee similarly protect neuron against H_2O_2-mediated apoptosis. Chlorogenic acid, a major phenolic phytochemical found in both caffeinated and decaffeinated coffee, also conferred protection against H_2O_2-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-$_X$L and inhibited pro-apoptotic cleavage of caspase-3 and pro-PARP,

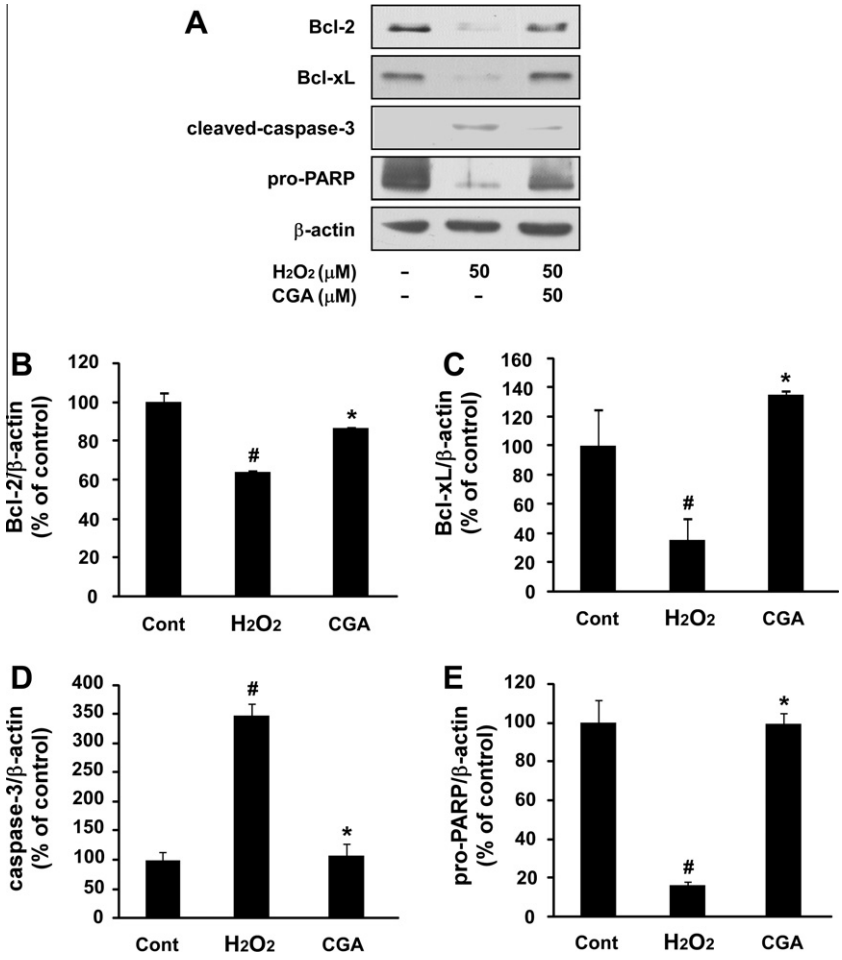

Fig. 4. Anti-apoptotic effects of chlorogenic acid (CGA) on H_2O_2-induced down-regulation of Bcl-2 and Bcl-xL as well as cleavage of caspase-3 and pro-PARP in primary neuronal culture. Cells were pretreated with chlorogenic acid (CGA) at 50 μM for 1 h followed by 50 μM H_2O_2 for 24 h. (A) The levels of Bcl-2, Bcl-xL, cleaved caspase-3, and cleaved pro-PARP were examined by Western blot analysis. β-Actin was measured to confirm uniform protein loading. The ratio of (B) Bcl-2/β-actin, (C) Bcl-xL/β-actin, (D) caspase-3/β-actin, and (E) pro-PARP/β-actin was determined by densitometry. Data are presented as means ± SE of three independent experiments. # Indicates significant difference at $p < 0.05$ comparing control vs. H_2O_2, and *, H_2O_2 vs. CGA.

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 (Riley and Workman, 1992; Talalay et al., 1995). NQO1 is a component for the plasma membrane redox system, which provides electrons for energy metabolism and recycling of antioxidants (Hyun et al., 2010). NQO1 maintains cellular levels of ubiquinol and vitamin E, two important biological antioxidants involved in the detoxification of ROS (Ross, 2004; Siegel et al., 1997). Moreover, NQO1 is expressed in neural cells and up-regulated in response to mitochondrial impairment and protect the cells against oxidative stress (Hyun et al., 2007, 2010; Rodriguez-Aguilera et al., 2000; Villalba and Navas, 2000).

Overexpression of NQO1 was found to protect neurons against amyloid-β cytotoxicity (Hyun et al., 2010). Cross-species microarray analysis suggested that NQO1 mediates neuroprotective pathways in aging and Alzheimer's disease (Lu et al., 2007). NQO1 also plays a critical role in protecting neuronal cells against dopamine or 6-hydroxydopamine-induced oxidative stress (Ross et al., 2000a; Spencer et al., 1998). Indeed, a recent observation suggested that polymorphism of NQO1 is associated with an increased risk of developing Parkinson's disease (Fong et al., 2007). It has been reported that coffee compounds can activate neuroprotective transcription factor Nrf2, which regulates the expression of antioxidant enzyme NQO1 (Trinh et al., 2010). The possible neuroprotection mediated by caffeinated coffee, decaffeinated coffee, and chlorogenic acid against H_2O_2 may be at least partly due to Nrf2-induced modulatory effects on NQO1 expression.

Bcl-2 and Bcl-$_X$L promote cell survival and down-regulation of Bcl-2 and Bcl-$_X$L occurs during apoptosis (Olie et al., 2002; Tsujimoto et al., 1997). The Bcl family of proteins inhibits the activation of the caspase-3 cascade and apoptosis (Zhao et al., 2003). 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) (Brauns et al., 2005; Lee et al., 2006). It has been reported that dicoumarol, a potent inhibitor of NQO1, decreases the protein level of Bcl-xL and potentiates to induce cytotoxicity (Buranrat et al., 2010). 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 (Weinreb et al., 2008). 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

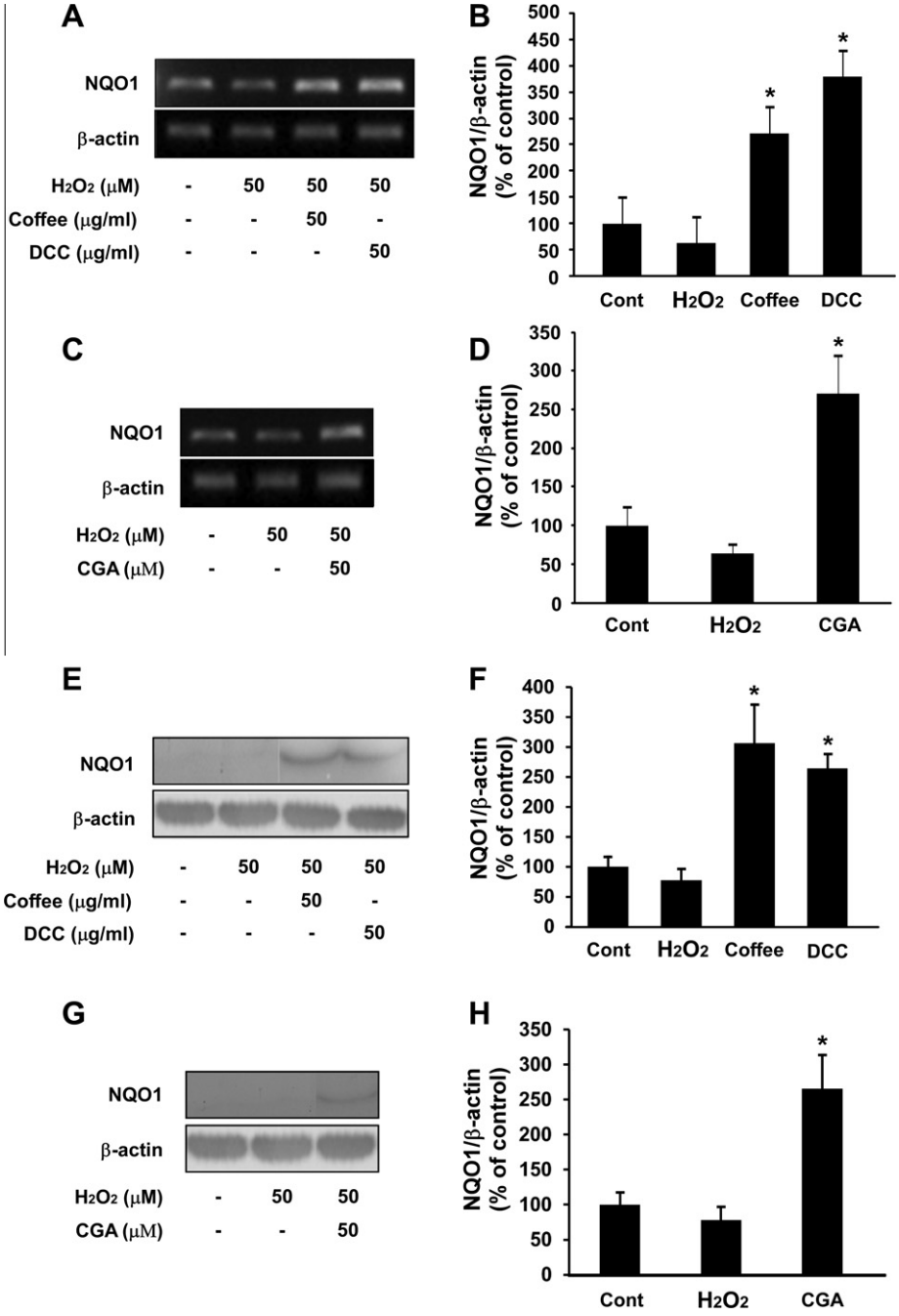

Fig. 5. Caffeinated coffee-, decaffeinated coffee- (DCC-), and chlorogenic acid (CGA)-mediated NQO1 expression in primary neuronal culture. Cells were pretreated with caffeinated coffee and decaffeinated coffee (DCC) at 50 μg/ml, and chlorogenic acid (CGA) at 50 μM for 1 h followed by 50 μM H_2O_2 for 24 h. (A and C) The mRNA levels of NOQ1 were examined by RT-PCR. β-Actin was measured to confirm uniform loading. (B and D) The ratio of NQO-1/β-actin was determined by densitometry. (E and G) The protein levels of NOQ1 were examined by Western blot analysis. β-Actin was measured to confirm uniform loading. (F and H) The ratio of NQO-1/β-actin was determined by densitometry. Data are presented as means ± SE of three independent experiments. * indicates significant difference at $p < 0.05$ comparing H_2O_2 vs. CGA.

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 (Kwon et al., 2010). 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 (Lapchak, 2007; Ohnishi et al., 2006). 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 (Chu et al., 2009).

The amount of chlorogenic acid in a 200 ml cup of coffee is about 70–350 mg (Clifford et al., 2007; Higdon and Frei, 2006). 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 ± 1.64 μM after oral ingestion of 190 ml of coffee drink containing 1068 ± 49 μmol (67.26 mg) chlorogenic acid (Monteiro et al., 2007). Studies on bioavailability after several months and years of chlorogenic acid-rich

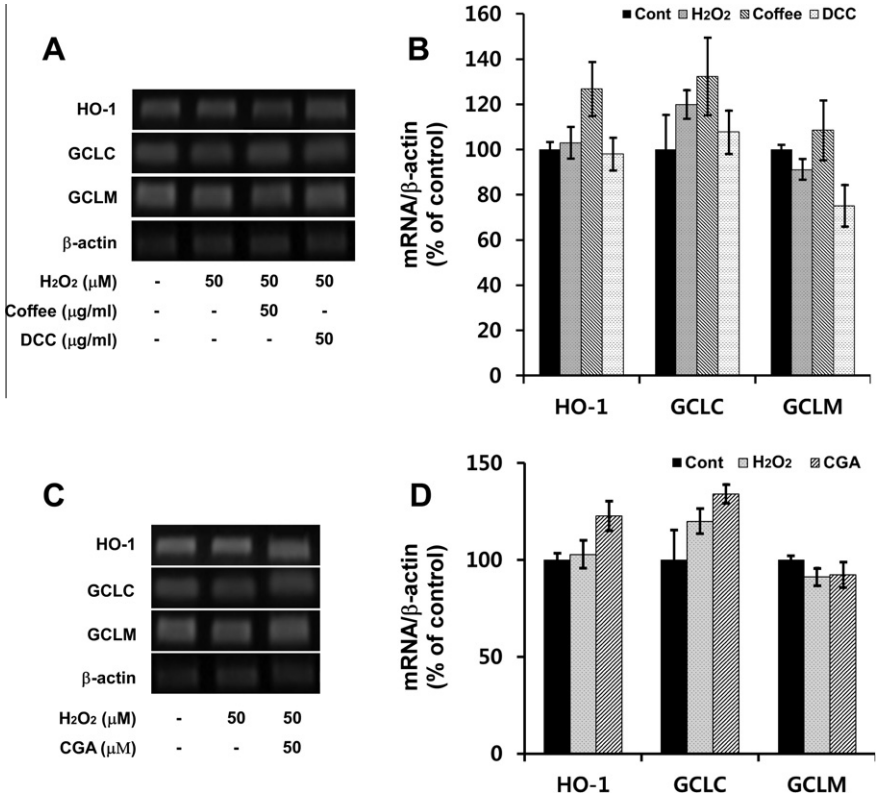

Fig. 6. Caffeinated coffee-, decaffeinated coffee- (DCC-), and chlorogenic acid (CGA)-mediated HO-1, GCLC and GCLM expression in primary neuronal culture. Cells were pretreated with caffeinated coffee and decaffeinated coffee (DCC) at 50 μg/ml, and chlorogenic acid (CGA) at 50 μM for 1 h followed by 50 μM H_2O_2 for 24 h. (A and C) The mRNA levels of HO-1, GCLC and GCLM were examined by RT-PCR. β-Actin was measured to confirm uniform loading. (B and D) The ratio of HO-1/β-actin, GCLC/β-actin and GCLM/β-actin was determined by densitometry. Data are presented as means ± SE of three independent experiments.

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 (Chu et al., 2009). Caffeic acid, present in coffee, has been shown to be neuron-protective *in vivo* under pathological conditions (Zhou et al., 2006). Kahweol and cafestol were also suggested as antioxidative and neuroprotective components in coffee (Hwang and Jeong, 2008; Trinh et al., 2010). 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_2O_2-induced apoptotic neuronal death and that chlorogenic acid might be largely responsible for these effects. This protection occurred through the inhibition of H_2O_2-induced down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X_L as well as the blockage of H_2O_2-induced pro-apoptotic cleavage of caspase-3 and pro-PARP in primary cortical neurons. We 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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