α- and β-Naphthoflavone synergistically attenuate H_2O_2-induced neuron SH-SY5Y cell damage

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Received November 6, 2015; Accepted November 11, 2016

DOI: 10.3892/etm.2017.4045

Abstract. Previous studies have demonstrated an association between neurological diseases and oxidative stress (OS). Naphthoflavone is a synthetic derivative of naturally occurring flavonoids that serves an important role in the treatment and prevention of OS-related diseases. The current study was designed to apply α- and β-Naphthoflavone individually and in combination to counteract the detrimental effects of OS on neurons *in vitro*. Neuronal SH-SY5Y cells were subjected to 20 μM H_2O_2, followed by exposure to 20 μM α-Naphthoflavone and/or 10 μM β-Naphthoflavone. Results indicated that α- and β-Naphthoflavone effectively antagonized the apoptosis-promoting effect of H_2O_2 on neuronal SH-SY5Y cells, and that β-Naphthoflavone significantly ($P<0.05$) reversed H_2O_2-inhibited cell viability. Notably, co-treatment of α- and β-Naphthoflavone reversed the H_2O_2-induced apoptosis rate elevation and cell viability reduction. Further analysis demonstrated that H_2O_2 inhibited the activities of antioxidant enzymes including catalase, superoxide dismutase and glutathione peroxidase, but this was reversed by the co-treatment with α- and β-Naphthoflavone and selectively enhanced by the treatment with α- or β-Naphthoflavone. H_2O_2-stimulated p38 mitogen-activated protein kinase activation was repressed following treatment with α- and/or β-Naphthoflavone, along with a decreased expression of the apoptosis-related factors and inhibited caspase-3 activation. In conclusion, co-treatment with α- and β-Naphthoflavone minimized H_2O_2-led neuron damage compared with treatment with α- or β-Naphthoflavone, suggesting a synergetic effect between α- and β-Naphthoflavone. This indicates that utilizing α- and β-Naphthoflavone together in the clinical setting may provide a novel therapeutic for neurological disease.

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Key words: α-Naphthoflavone, β-Naphthoflavone, antioxidant enzyme activity, oxidative stress, p38 mitogen-activated protein kinase

Introduction

Oxidative stress (OS) is an important contributor to the damage induced by acute ischemic stroke and is a potent etiologic factor for chronic neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis and amyotrophic lateral sclerosis (1,2). The implication of OS in neurological diseases is associated with its effect on neurocytes; the structural and functional characteristics present in neurocytes include easily peroxidizable unsaturated fatty acids, high oxygen consumption rate and relative paucity of antioxidant enzymes, resulting in the vulnerability of neurocytes to OS (2). Furthermore, the incidence of OS and the opportunity of neurocytes exposed to OS are increased by exposure to environmental toxins (3), deficient antioxidants present in the diet source (4) and pathological conditions such as diabetes (5). An increase in OS is caused by excessive production of oxidative radical species (ROS), leading to the endogenous antioxidant defenses being overwhelmed (6). ROS exert a strong oxidative property and may result in cell membrane injury, mitochondrial dysfunction, protein misfolding and DNA destruction, finally leading neurocyte apoptosis (7).

OS-induced neurocyte apoptosis is a primary contributor to neurological disease with OS in the reoxygenation/reperfusion setting of stroke constituting a major driving force for neurocyte apoptosis (8-10). The immediate supplement of exogenous antioxidants has been demonstrated to decrease neuronal mortality and ease the burden of stroke *in vivo* and *in vitro* (8-10). By contrast, OS derived from microglial activation in PD induces neurocyte apoptosis and consequently assists the degeneration of dopaminergic neurons that is a marked pathological feature of PD (11,12). In AD, the incidence of neuronal apoptosis is notably elevated, which is associated with the induction of OS production through the disruption of ceramide metabolism and accumulation of amyloid β-peptides (13). Neurocytes are unable to be regenerated following apoptosis and the deficit of neurocytes may trigger typical characteristics of neurological diseases including memory loss, cognitive impairment and motor

disturbance. Therefore, counteracting the detrimental effects of ROS, especially ROS-induced neuronal apoptosis, is an important approach in treating neurological diseases (10-13).

A number of botanical extracts, specifically those rich in flavonoids, confer neuroprotective effects via the inhibition of OS generation and inhibiting OS-mediated deleterious actions (14). Lin *et al* (15) demonstrated that the *Wedelia chinensis* extract effectively alleviates tertbutyl hydroperoxide-induced oxidative damage in neuronal PC12 cells and D-galactose-induced lipid peroxidation in the cerebral cortex of mice. *Ratanasampil* contains numerous Chinese herbs that have been demonstrated to have neuroprotective effects on OS-induced neuronal SH-SY5Y cell damage (16). In the present study, Naphthoflavone, a synthetic derivative of naturally occurring flavonoids, observed in *Passiflora incarnata Linn* (17), was assessed. Naphthoflavone has the potential to be a readily available and cheap therapeutic agent, if validated to be effective against neurological diseases. Naphthoflavone only contains two structural isomers, α-Naphthoflavone and β-Naphthoflavone, which is convenient for the investigation of their biological functions. By contrast, many botanical extracts, despite their neuroprotective effects have been identified to contain a number of different types of bioactive components (18). Therefore, the specific components of botanical extracts that are neuroprotective and their underlying mechanisms are typically poorly understood, resulting in restricted application of many botanical extracts in clinical settings. It has been demonstrated that Naphthoflavone upregulates the expression or activity of antioxidant-associated enzymes, including glutathione peroxidase (GPx), quinone oxidoreductase-1, glutathione transferase and heme oxygenase-1 (19-22), and represses ROS-producing enzymes such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (23). This suggests a potential effect exerted by Naphthoflavone in antioxidation. Currently, limited studies have assessed the role of Naphthoflavone in neurons that suffer from OS. Therefore, the present study aimed to administer α- and β-Naphthoflavone individually and combined to determine their capacity to counteract the detrimental effects of OS on neurons *in vitro*.

Materials and methods

Agents and cell culture. α- and β-Naphthoflavone were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The human neuron SH-SY5Y cells obtained from the American Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Hyclone) and 100 U/ml streptomycin and penicillin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) in a 5% CO_2 incubator at 37˚C.

Cell viability assay. The cell viability was evaluated using a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) using the following method. SH-SY5Y cells were seeded in 96-well plates at $1x10^4$ cells/well and cultured in DMEM without FBS at 37˚C for 48 h. The cells were subjected to H_2O_2 at concentration of 0-320 μM for 24 h to evaluate the impairment. In an independent experiment, the cells underwent 20 μM H_2O_2 for 24 h followed by treatments with α- and β-Naphthoflavone, alone or in combination. SH-SY5Y cells that were subjected to 20 μM H_2O_2 for 24 h were subsequently treated with 0.5 μM SB203580 (Selleck Chemicals, Houston, Texas, USA), a p38MAPK-specific inhibitor, for 24 h. Finally, 10 μl CCK-8 solution was added to each well followed by an additional 5-8 h of incubation at 37˚C. The optical density was then measured using a microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA) at a wavelength of 450 nm.

Apoptosis rate analysis. The apoptosis rate was evaluated using the Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection kit I (cat. no. 550475; BD Biosciences, San Jose, CA, USA). A total of 5 μl Annexin V-FITC and 5 μl propidium iodide were added to SH-SY5Y cells (~$2x10^5$) that were treated with Naphthoflavone and incubated at room temperature for 15 min in the dark. The extent of apoptosis was analyzed with a dual laser FACSCalibur flow cytometer (BD Biosciences) and estimated using the ModFit LT™ software version 2.0 (Verity Software House, Topsham, ME, USA).

Total antioxidant capacity (TAC) and malondialdehyde (MDA) measurement. The supernatant of the cells was lysed using a radioimmunoprecipitation assay (RIPA) buffer (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) in order to measure the TAC using the azino-diethyl-benzthiazoline sulfate (ABTS) method (24). Total antioxidant capacity detection kit was purchased from Nanjing KeyGen Biotech Co., Ltd. Incubation of ABTS with H_2O_2 and a peroxidase (metmyoglobin) produces a blue-green radical cation, $ABTS^+$. Antioxidants in the sample suppress this color production proportionally to their concentration. Trolox (Nanjing KeyGen Biotech Co., Ltd.), a water-soluble vitamin E analogue was used to standardize the system. The results were expressed as μmol Trolox equivalent/protein concentration of plasma supernatant of lysed cells. In the MDA assay, 2 ml 0.6% thiobarbituric acid (Nanjing KeyGen Biotech Co., Ltd.) was added to 2 ml supernatant in a 10 ml tube. This tube was incubated in boiling water for 15 min and placed on ice to cool down prior to the optical density being measured at 532 nm via a microplate reader (ELx800; BioTek Instruments, Inc.). The results were expressed in nmol MDA/g protein.

Antioxidant enzyme activity assay. Catalase (CAT), superoxide dismutase (SOD) and GPx activity detection kits were all obtained from Beyotime Institute of Biotechnology (Haimen, China). In regard to CAT, cell homogenates were placed in a cuvette that had contained 250 mM hydrogen peroxide (H_2O_2) for 1-5 min. The remaining hydrogen peroxide was coupled with a chromogenic substrate (Beyotime Institute of Biotechnology) to generate a red product, N-4-antipyryl-3-chloro-5-sulfonate-p-benzoquinonemonoimine, which has a maximal absorption at 520 nm. Testing the remaining hydrogen peroxide enabled the quantity of H_2O_2 that reacted with CAT to be calculated and in turn determine the CAT activity. In the present study, SOD was present in the samples; this inhibited the process of superoxide transforming WST-8, a 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (Nanjing KeyGen Biotech Co., Ltd.), to a stable water-soluble WST-8 formazan. The

latter may be evaluated by testing the optical density at 450 nm in order to determine SOD activity. The determination of GPx activity was based on the principle that NADPH continually diminishes in the cycle of GPx (6), transforming reduced glutathione to oxidized glutathione, which is reversed by glutathione reductase. Detecting reduced NADPH in absorbance at 340 nm may indirectly estimate the activity of GPx.

Western blot analysis. The cells were washed in PBS and lysed with RIPA buffer (50 mM Tris-buffer, 170 μg/ml leupeptin, 150 mM sodium chloride, 5 mM EDTA, 1 mM sodium orthovanadate, 1% NP-40, 81 μg/ml aprotinin, 0.5% deoxycholic acid, and 100 μg/ml phenylmethylsulfonyl fluoride, all these agents were purchased from Sigma-Aldrich; Merck Millipore, containing phosphatase inhibitors (okadaic acid; cat. no. ab120375; Abcam, Cambridge, UK). Protein concentration was measured using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). A 10% SDS-PAGE gel loaded with ~20 μg protein from the cell lysis buffer was used to separate the proteins; these were then transferred onto polyvinylidene fluoride membranes (Merck Millipore) that were initially soaked in absolute methanol. The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing Tween for 1 h. Proteins were probed with the following primary antibodies: anti-p38 mitogen-activated protein kinase (p38MAPK; 1:1,000, ab27986), anti-p38MAPK (phospho T180 + Y182; 1:800, ab195049), anti-cytochrome *c* (Cyt C; 1:200, ab53056), anti-caspase-3 (1:500, ab47131) purchased from Abcam. Anti-p38MAPK (phospho T322; 1:800, AP50174; Abgent, Inc., San Diego, CA, USA), anti-Bax (1:300, sc-493; Santa Cruz Biotechnology, Inc., La Jolla, CA, USA), and anti-GAPDH (1:5,000, ab16884; Abcam) at 37˚C for 2 h. Following three washes, the membranes were incubated with anti-rabbit IgG-horseradish peroxidase-conjugated secondary antibodies (1:20,000; cat. no. 611-903-002, Rockland Immunochemicals, Inc., Plottstown, PA, USA) at room temperature for 1 h. The blots on the membranes were scanned to quantify the optical density (Odyssey Image-forming System; LI-COR Biotechnology, Lincoln, NE, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean following three independent experiments. Data from these experiments were analyzed using SPSS software, version 12.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance with post hoc testing was used for multiple comparisons between each group. Significant differences were determined as $P<0.05$.

Results

Naphthoflavone attenuates H_2O_2-induced cell viability reduction and apoptosis elevation. H_2O_2 is recognized as a powerful oxidant and is commonly used to establish the OS model in a number of cell types. In the present study, the alteration of SH-SY5Y cell viability following cell exposure to H_2O_2 at concentration from 0-320 μM for 24 h was investigated. The cell viability was decreased by H_2O_2 in a dose-dependent manner, with the half cell viability loss at 20 μM H_2O_2 (Fig. 1A). Following the exposure to 20 μM H_2O_2 for 24 h, H_2O_2 was removed and cells were cultivated for an additional 24 h in the presence or absence of Naphthoflavone. The dose-response curves for the H_2O_2-pretreated cells cultivated with different doses of α- and β-Naphthoflavone are presented in Fig. 1B and C, respectively. The H_2O_2-inhibited cell viability increased in a dose-dependent manner by α-Naphthoflavone prior to the cell viability reaching a plateau at the dose of 20 μM α-Naphthoflavone (Fig. 1C). By contrast, the cell viability was increased with β-Naphthoflavone concentration raised from 0-10 μM, followed by a slight drop (Fig. 1C). Therefore, 20 μM α-Naphthoflavone and 10 μM β-Naphthoflavone, caused the maximal effect on cell viability recovery and were used individually and in combination in subsequent experiments of the present study to counteract 20 μM H_2O_2-induced detrimental effects. Results presented in Fig. 1D indicate that 20 μM H_2O_2 administered to the positive control (PC) cells induced a significant reduction of the cell viability compared to negative control (NC) cells without any treatment ($P<0.05$). This reduced cell viability was significantly increased by 10 μM β-Naphthoflavone ($P<0.05$ vs. PC), but not 20 μM α-Naphthoflavone ($P=0.064$; Fig. 1D). Furthermore, a significant increase in cell viability was observed following co-treatment with 20 μM α-Naphthoflavone and 10 μM β-Naphthoflavone compared with PC group ($P<0.05$). The co-treatment recovered the cell viability to a level close to that of the NC group.

As indicated by the apoptosis rate assay, H_2O_2 administration increased the apoptosis rate of SH-SY5Y cells in the PC group to 24.1%, ~5-fold increase compared with the NC group ($P<0.01$). Following this treatment, the addition of α- and β-Naphthoflavone, respectively, significantly decreased the apoptosis rate in comparison to the PC cells ($P<0.05$; Fig. 2A and B). Notably, co-treatment with α-Naphthoflavone and β-Naphthoflavone significantly reduced the apoptosis rate compared with the PC group, to 7.2% ($P<0.01$).

Naphthoflavone reverses H_2O_2-induced TAC decrease and MDA increase. To understand the H_2O_2-induced oxidative effect and potential antioxidant function of Naphthoflavone, the oxidative/antioxidative status of SH-SY5Y cells following different treatments was assessed via TAC and MDA assays. The TAC of SH-SY5Y cells (PC group) was significantly decreased following exposure to 20 μM H_2O_2 ($P<0.01$, Fig. 3A). Both α- and β-Naphthoflavone significantly reversed the H_2O_2-induced reduction in TAC compared with the PC group ($P<0.05$). Combined treatment with α- and β-Naphthoflavone had the most marked effect in on TAC, which significantly increased TAC in comparison to the PC group ($P<0.05$). MDA is regarded as a typical and sensitive biochemical marker following cell exposure to ROS and its accumulation reflects the extent of OS and indirectly that of cellular antioxidant capacity (25). In the current study, MDA in cells subjected to H_2O_2 (PC group) increased to ~2.5-fold higher compared with NC cells, reaching 13.24 μmol/mg protein ($P<0.05$, Fig. 3B). Post-treatment with α- and β-Naphthoflavone decreased MDA to 10.24 and 9.01 μmol/mg protein, respectively, which was a significant reduction compared with the PC group ($P<0.05$). Co-treatment with α- and β-Naphthoflavone reduced MDA to 6.74 μmol/mg, which was a significantly reduction compared with the PC ($P<0.05$).

Antioxidant enzyme activities are upregulated by Naphthoflavone. As presented in Fig. 3C-E, exposure to H_2O_2

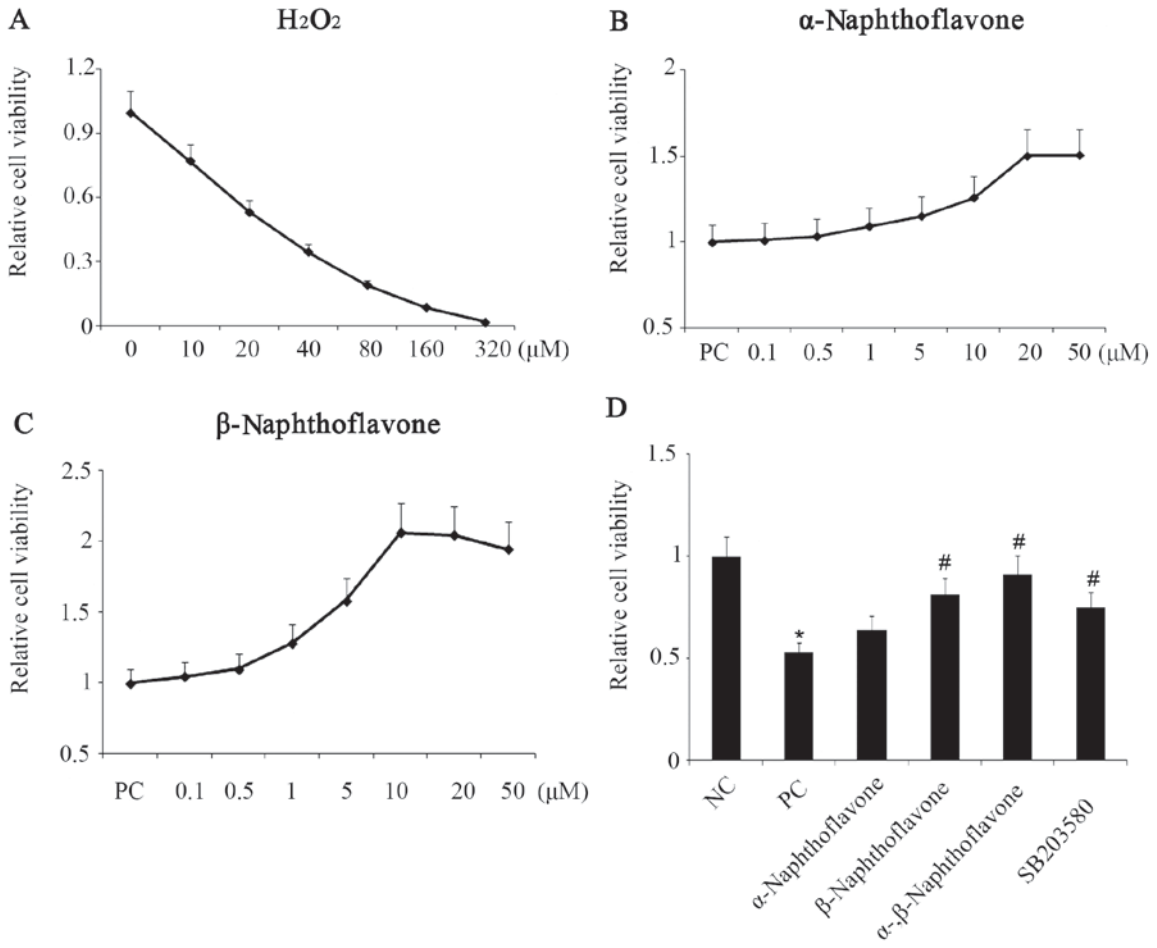

Figure 1. Naphthoflavone reverses H_2O_2-inhibited neuron SH-SY5Y cell viability. (A) Neuron SH-SY5Y cell viability decreased by H_2O_2 in a dose-dependent manner, with half the cell viability loss at 20 μM H_2O_2. (B) H_2O_2-inhibited neuron SH-SY5Y cell viability increased in a dose-dependent manner with α-Naphthoflavone, until the viability reached the plateau at a dose of 20 μM α-Naphthoflavone. (C) H_2O_2-inhibited neuron SH-SY5Y cell viability increased with β-Naphthoflavone concentration, 0-10 μM, followed by a slight decrease. (D) Neuron SH-SY5Y cell viability following different treatments. α- and β-Naphthoflavone at 20 and 10 μM, respectively, were used individually and in combination following exposure of neuron SH-SY5Y cells to 20 μM H_2O_2. *P<0.05 vs. NC; #P<0.05 vs. PC (n=8). NC, negative control, cells without any treatment; PC, positive control, cells treated with 20 μM H_2O_2 only; α-, β-Naphthoflavone, co-treatment with 20 μM α-Naphthoflavone and 10 μM β-Naphthoflavone; SB203580, p38MAPK inhibitor; H_2O_2, hydrogen peroxide.

decreased the activities of CAT, SOD and GPx by 82, 79 and 63%, compared with their respective NC values (P<0.01, P<0.01 and P<0.05, respectively). Post-treatment with α-naphthoflavone significantly increased SOD activity compared with the PC group (P<0.05; Fig. 3D), but only marginally restored the enzyme activities of CAT and GPx (Fig. 3C and E). Post-treatment with β-Naphthoflavone significantly increased CAT and SOD enzyme activities compared with the PC group (Fig. 3C and D; P<0.05). Combined treatment with α- and β-Naphthoflavone produced the most marked effect, which predominantly elevated SOD enzyme activity compared to PC (P<0.01; Fig. 1D), as well as increased CAT and GPx activities significantly compared with the PC group (Fig. 3C and E; P<0.05).

Naphthoflavone attenuates H_2O_2-induced damage via p38MAPK inhibition. Previous studies have demonstrated that H_2O_2 activates p38MAPK-mediated signaling to induce neuronal apoptosis (26). In the present study, SH-SY5Y cells exposed to H_2O_2 exhibited a 2.2-fold increase in p38MAPK phosphorylation at the sites of Thr180 and Tyr182 (P<0.05, Fig. 4A), although non-phosphorylated p38MAPK and phosphorylated p38MAPK at site of Thr322 were almost unaffected, as assessed by western blot analysis (Fig. 4A). By contrast, H_2O_2 administration markedly increased the expression of apoptosis-associated factors including Bax (P<0.01, Fig. 4B), Cyt C (P<0.01, Fig. 4C) and the ratio of cleaved to non-cleaved caspase-3 (P<0.01, Fig. 4D). Subsequent addition of a p38MAPK-specific inhibitor (SB203580) effectively reversed the elevation of p38MAPK phosphorylation on Thr180 and Tyr182 (P<0.05 vs. PC, Fig. 4A), and is associated with the reduction in the rate of apoptosis identified in Bax and Cyt C expression (P<0.05 vs. PC, Fig. 4B and C), the cleaved ratio of caspase-3 (P<0.01 vs. PC, Fig. 4D) and SH-SY5Y cells (P<0.05 vs. PC; Fig. 2). The data from the present study suggest that H_2O_2-induced p38MAPK activation facilitates neuron SH-SY5Y cell apoptosis, as previously considered (26). There is a limited data regarding the antioxidant role of SB203580**;** however, in the present study the inhibitor exhibited an antioxidant capacity by effectively reversing the H_2O_2-mediated increase of MDA (P<0.05; Fig. 3B) while H_2O_2-decreased CAT (P<0.05; Fig. 3C) and SOD activities (P<0.05; Fig. 3D).

Post-treatment with Naphthoflavones also repressed the p38MAPK phosphorylation at the Thr180 and Tyr182 sites and

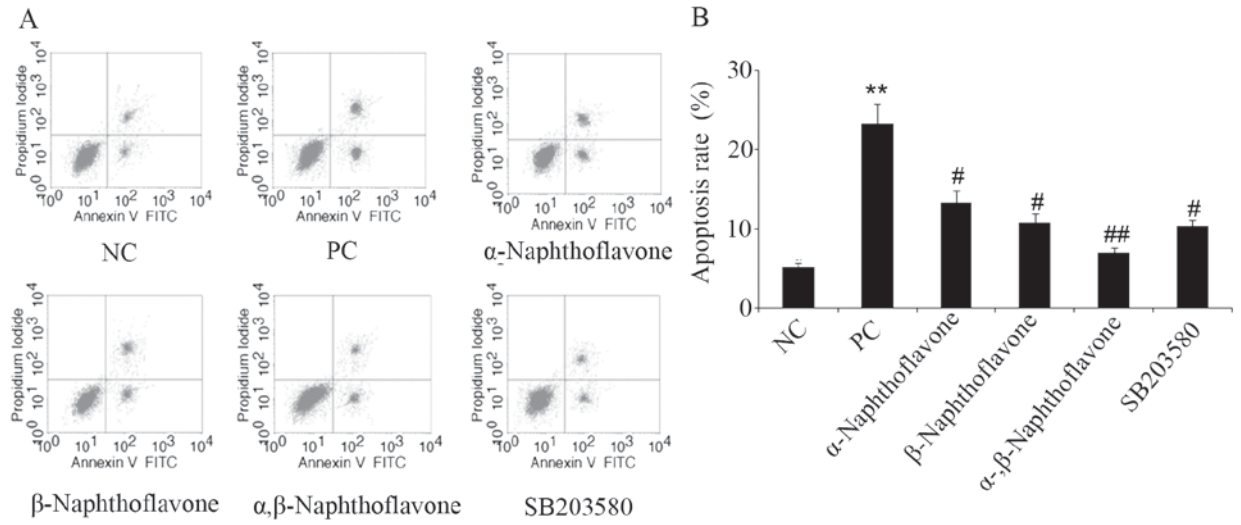

Figure 2. Naphthoflavone decreases elevation of apoptosis in H_2O_2-induced elevation in neuron SH-SY5Y cells. α- and β-Naphthoflavone at a dose of 20 and 10 μM, respectively, were used individually and in combination following exposure of neuron SH-SY5Y cells to 20 μM H_2O_2. Apoptosis rate was (A) detected by flow cytometry and (B) quantified. *P<0.05, **P<0.05 vs. NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. PC (n=4). NC, negative control, cells without any treatment; PC, positive control, cells treated with 20 μM H_2O_2 only; α-, β-Naphthoflavone, co-treatment with 20 μM α-Naphthoflavone and 10 μM β-Naphthoflavone; SB203580, p38MAPK inhibitor; H_2O_2, hydrogen peroxide.

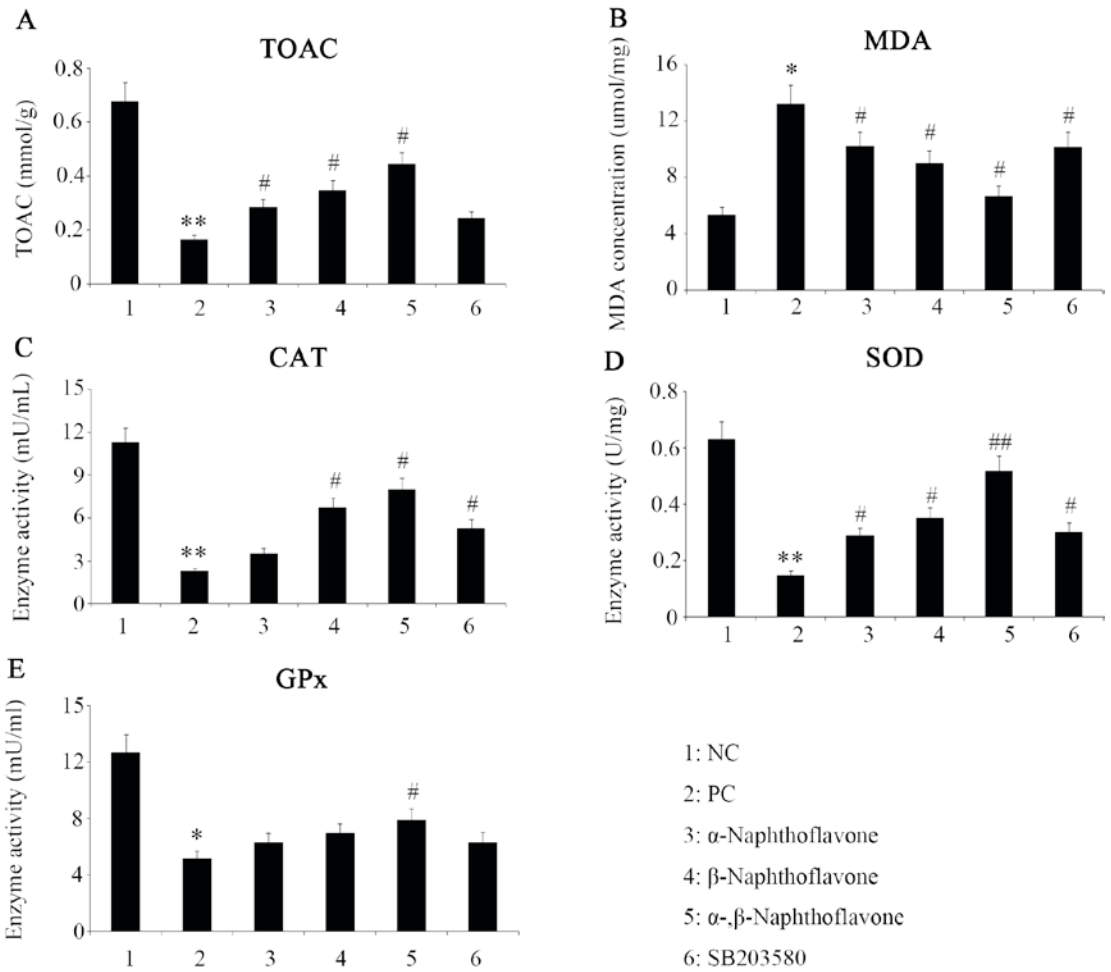

Figure 3. Oxidative or antioxidative status of neuron SH-SY5Y cells following different treatments. α- and β-Naphthoflavone at a dose of 20 and 10 μM, respectively, were used individually and in combination following exposure of neuron SH-SY5Y cells to 20 μM H_2O_2. The oxidative or antioxidative status of neuron SH-SY5Y cells following treatment with (A) TAC, (B) MDA, (C) CAT, (D) SOD and (E) GPx. *P<0.05, **P<0.05 vs. NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. PC (n=4). NC, negative control, cells without any treatment; PC, positive control, cells treated with 20 μM H_2O_2 only; α-, β-Naphthoflavone, co-treatment with 20 μM α-Naphthoflavone and 10 μM β-Naphthoflavone; SB203580, p38MAPK inhibitor; TAC, total antioxidant capacity; MDA, malondialdehyde; CAT, catalase; SOD, superoxide dismutase; GPx, glutathione peroxidase.

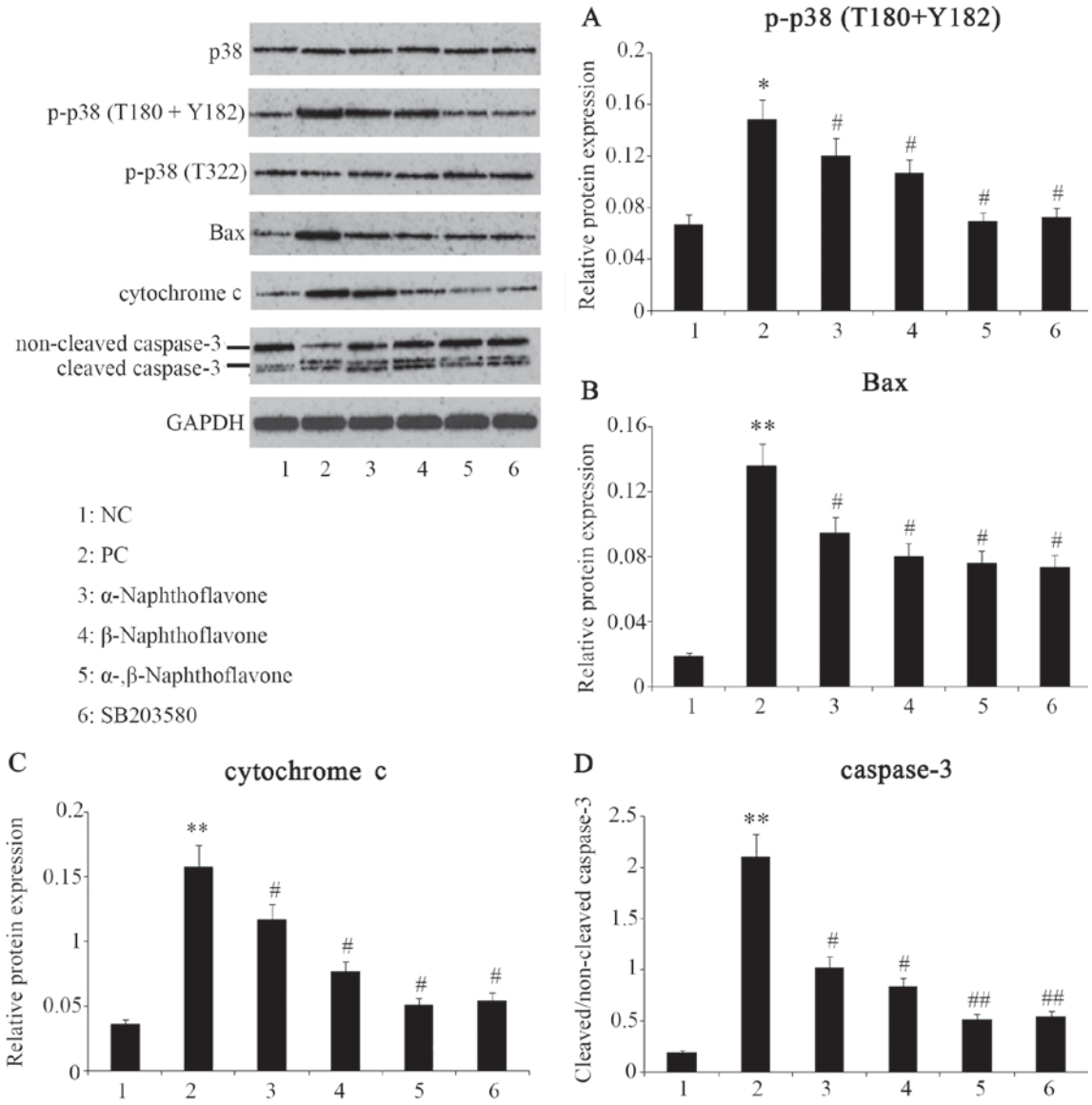

Figure 4. Expression levels of proteins following different treatments. α- and β-Naphthoflavone at a dose of 20 and 10 μM, respectively, were used individually and in combination following exposure of neuron SH-SY5Y cells to 20 μM H_2O_2. (A) Western blot analysis indicates p38MAPK phosphorylation at Thr180 and Tyr182. The protein expression of (B) Bax (C) cytochrome c and (D) caspase-3 following different treatments. *P<0.05, **P<0.05 vs. NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. PC (n=4,). NC, negative control, cells without any treatment; PC, positive control, cells treated with 20 μM H_2O_2 only; α-, β-Naphthoflavone, co-treatment with 20 μM α-Naphthoflavone and 10 μM β-Naphthoflavone; SB203580, p38MAPK inhibitor; p-p38, phosphorylated p38; H_2O_2, hydrogen peroxide.

this inhibitory effect, in order of least to most marked, was exerted by α-Naphthoflavone (P<0.05 vs. PC), β-Naphthoflavone (P<0.05 vs. PC) and combined α- and β-Naphthoflavone (P<0.05 vs. PC; Fig. 4A). Additionally, α-Naphthoflavone decreased the expression of Bax and Cyt C and cleaved ratio of caspase-3 (P<0.05 vs. PC). These effects were also exhibited by β-Naphthoflavone, which was more effective than α-Naphthoflavone, specifically in the inhibition of Cyt C expression. By contrast, combined utilization of α- and β-Naphthoflavone minimized Bax and Cyt C expression and the ratio of cleaved caspase-3 (P<0.05, P<0.05 and P<0.01 vs. PC, respectively).

Discussion

Naphthoflavone has been documented to alleviate and confer protection against deleterious effects induced by OS. Previous studies have indicated that α-Napthoflavone inhibits OS and serves potential roles is estrogen-induced breast carcinogenesis and ultraviolet-led human skin aging (27,28). β-Naphthoflavone has been demonstrated to protect mice against aristolochic acid-I-induced acute kidney injury, and to attenuate hyperoxic lung injury in premature infants primarily via mitigating OS (29,30). In the present study, α- and β-Naphthoflavone were identified to effectively antagonize the apoptosis-promoting effect of H_2O_2 on neuronal SH-SY5Y cells. β-Naphthoflavone significantly reversed H_2O_2-inhibited the cell viability (P<0.05). Naphthoflavone was represented as a toxic substance in a previous study (31); however, the harmful effect of Naphthoflavone was not observed in the concentration range of α- o β-Naphthoflavone in the present study. Co-treatment with α- and β-Naphthoflavone abrogated the elevation of H_2O_2-induced apoptosis rate and cell viability reduction, which suggests a synergetic effect between α- and β-Naphthoflavone in these respects. This advocates

the combined application of α- and β-Naphthoflavone in the clinical settings of neurological disease treatment.

OS causes structural and functional damage to neurons, H_2O_2-induced apoptosis mortality. As a frequent event in nervous system, it is therefore associated with a wide range of neurological diseases (1,2). OS is an important therapeutic target, and the supplement of exogenous antioxidants has been validated to be an effective approach to prevent and manage neurological diseases clinically (13-16). In the present study, neuronal SH-SY5Y cells underwent OS following exposure to H_2O_2, as indicated by TAC and MDA assays, and post-treatment with α-Naphthoflavone and/or β-Naphthoflavone effectively alleviated H_2O_2-induced OS.

A notable property of Naphthoflavone is an ablity to modulate antioxidant enzyme activity. It has been suggested that the antioxidant function exerted by natural flavones is completely dependent on their reducibility, derived from specific phenolic hydroxyl groups in their molecular structure (32). Evidence from Yang *et al* (6) indicates that natural flavones from grape seed extract elevate the activities of CAT, SOD and GPx, and mitigate the H_2O_2 insult to muscle cells, further suggesting that flavones serve an antioxidant role. It has been demonstrated previously that β-Naphthoflavone serves as a modulator of antioxidant enzyme activity (19). However, a previous study has indicated that antioxidant enzyme activity is unaffected following treatment with certain flavones (33). Thus, the present results indicate that the ability of antioxidant enzyme activity regulation is restricted in a number of flavones. In the current study, H_2O_2-inhibited CAT and SOD activities were promoted by β-Naphthoflavone. Furthermore, α-Naphthoflavone induced a compensatory increase in SOD activity. The activity of GPx was not significantly recovered by α- or β-Naphthoflavone, whereas it was effectively reversed by co-treatment with α- and β-Naphthoflavone. It has been demonstrated that antioxidant enzyme activity regulated by certain flavones potentially results in flavones stimulating or inhibiting aryl hydrocarbon receptors and subsequently regulating signaling mediated by nuclear factor erythroid 2-related factor, MAPK and peroxisome proliferator-activated receptors (6). This was partly supported by the results of the present study as SB203580 interfered with p38MAPK activation, resulting in the alteration of CAT and SOD enzyme activity, which may further explain why the addition of SB203580 may increase TAC and decrease MDA.

It has been indicated that OS-led neuronal apoptosis represents a major mechanism for the development and progression of neurological diseases (11-13) and OS-led neuronal apoptosis is at least in part dependent upon p38MAPK-mediated signaling. Park *et al* (26) demonstrated that the phosphorylation level of p38MAPK is markedly increased in SH-SY5Y cells following H_2O_2 stimulation, but those of extracellular signal regulated kinase and c-Jun N-terminal were not affected. Furthermore, increased p38MAPK phosphorylation is associated with an increase in the rate of SH-SY5Y cell apoptosis, which was eradicated following treatment with SB203580, a p38MAPK specific inhibitor (26). A similar outcome was observed in the present study and it was observed that OS-led neuronal apoptosis relies on p38MAPK phosphorylation at two sites (Thr180 and Tyr182), rather than at Thr322. α- and β-Naphthoflavone repressed p38MAPK phosphorylation at Thr180 and Tyr182, suggesting that α- and β-Naphthoflavone inhibition of H_2O_2-led SH-SY5Y cell apoptosis is partly dependent on p38MAPK signaling. However, it is unclear whether Naphthoflavone is inhibiting p38MAPK activation completely depends on repressing OS and its action on p38MAPK phosphorylation, or only partly depends. The latter suggests that Naphthoflavone may potentially inhibit p38MAPK activation through direct interference of its upstream signaling. To understand the mechanisms involved, future experiments to determine how dependent cell apoptosis in SH-SY5Y cells is on p38MAPK signaling are required.

Under OS conditions, numerous apoptosis-associated factors were upregulated or activated. Upregulated Bax and the released Cyt C from the mitochondria are tightly associated to mitochondrial dysfunction-mediated apoptosis (34). Conventional western blot assays are unable to examine proteins present in the mitochondria as the organelle is not lysed in this setting, and lysing mitochondria requires more specific conditions. However, a clear upregulation of Cyt C observed in the western blot analysis in the present study indicates that Cyt C was released from the mitochondria in large quantities. Caspase-3 activation is dependent on the cleavage of its inactive form, and is widely regarded as a key step in apoptosis execution (34). Notably, α- and β-Naphthoflavone individually and synergistically suppressed the expression of Bax and Cyt C and Caspase-3 activation, further confirming that Naphthoflavone may inhibit OS-led neuronal apoptosis.

The present results indicate that β-Naphthoflavone is more effective against OS-led neuron damage compared with α-Naphthoflavone. α-Naphthoflavone only induced a minor change to H_2O_2-inhibited SH-SY5Y cell viability, but it was markedly reversed by β-Naphthoflavone. Furthermore, the post-treatment with β-Naphthoflavone led to a reduced apoptosis rate compared with α-Naphthoflavone post-treatment. β-Naphthoflavone is more efficient at upregulating the activity of CAT, as well as inhibiting Cyt C released from the mitochondria compared with α-Naphthoflavone. Further differences between the mechanisms underlying α- and β-Naphthoflavone against OS-led neuron damage may potentially exist, as combined α- and β-Naphthoflavone had a more marked effect in this regard compared with α- or β-Naphthoflavone individually. Moreover, increasing doses did not continue to promote cell viability once the doses of α- and β-Naphthoflavone reached 20 and 10 μM, respectively. An improved understanding of the mechanisms by which α- and β-Naphthoflavone counteract OS-led neuron damage is necessary in future study. Furthermore, co-treatment with α- and β-Naphthoflavone abrogated H_2O_2-induced apoptosis rate elevation and cell viability reduction. This suggests a synergetic effect between α- and β-Naphthoflavone. Further analysis demonstrated that the activities of antioxidant enzymes including CAT, SOD and GPx, were inhibited by increased H_2O_2, but enhanced by α- and/or β-Naphthoflavone. H_2O_2-stimulated p38MAPK activation was repressed by α- and/or β-Naphthoflavone, along with a decreased expression of the apoptosis-related factors and inhibited caspase-3 activation.

In conclusion, co-treatment with α- and β-Naphthoflavone minimized H_2O_2-induced neuron damage compared with treatment with α- or β-Naphthoflavone individually. Therefore, it is advocated that utilizing α- and β-Naphthoflavone together in the clinical setting of neurological disease treatment may be beneficial.

Acknowledgements

The present study was supported by the Research Project of Health and Family Planning Commission of Hunan Province (grant no. B2016196).

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