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Luteolin from Purple Perilla mitigates ROS insult particularly in primary neurons

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Abstract

Increased attention has been paid to the role of oxidant/antioxidant imbalance in neurodegenerative process and pharmaceutical neuroprotective interventions. Food-derived compound luteolin possesses multitarget actions including reactive oxygen species (ROS)-scavenging activity in cultured human endothelial cells or permanent immature rat oligodendrocytes. This study aims to elucidate whether luteolin has a neuroprotective tendency toward ROS-insulted neural cells. The present results showed that luteolin, isolated from the ripe seed of *Perilla frutescens* (L.) Britt., markedly reversed hydrogen peroxide-induced cytotoxicity in primary culture cortical neurons but not in cultured human neuroblastoma cells. Upon the ROS-insulted primary neurons, luteolin concentration-dependently enhanced neuronal cell survival with efficacy higher than and potency similar to vitamin E. Additionally, luteolin significantly attenuated the increase in ROS production and prevented the decreases in activities of mitochondria, catalase, and glutathione in ROS-insulted primary neurons. Thus, luteolin functions by neuroprotection possibly through a rebalancing of pro-oxidant-antioxidant status. This agent points to possible interventions for preventing neurodegenerative diseases such as cerebral ischemia, Parkinson's disease, and Alzheimer's disease, as well as for improving brain aging.

Keywords: Antioxidation; Hydrogen peroxide; Luteolin; Neuroprotectant; Oxidant-antioxidant balance; Reactive oxygen species

1. Introduction

The high morbidity, high socioeconomic costs, and lack of treatment-specific medication are key factors that define the relevance of neurodegenerative disorders to human health and the importance of developing neuroprotective pharmaceuticals in the clinical attenuation of neurodegeneration. The involvement of reactive oxygen species (ROS) has been implicated in the pathogenesis and/or progression of Parkinson's disease, ischemic and postischemic brain cell damage, dementia, and brain aging (Klein and Ackerman, 2003; Swerdlow, 2007; Yoshioka et al., 2002). Moreover, antioxidants have a propensity to block or delay the apoptosis of neuronal cells and thereby function by prevention of neurodegenerative disorders (Zhang et al., 2006). Under experimental conditions, several neuroprotectants have been uncovered, e.g., pure scavenger molecules such as boldine (Schmeda-Hirschmann et al., 2003), N-methyl-D-aspartic acid receptor blockers (Vural et al., 2009), and chain-breaking vitamin E (VE) (Hensley et al., 2004), each of which, however, acts via a 1-target mechanism and fails clinically to protect neurons against ROS stress/excitotoxicity. During the last few years, cellular oxidant/antioxidant balance has become the subject of intense study (Calabrese et al., 2006), and novel cytoprotective strategies responding to oxidant/antioxidant imbalance may effectively delay the neurodegenerative process (Calabrese et al., 2000, 2006). Besides, protection of neurons against diverse pathogenic factors has

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recently been proved a useful approach to clinically limiting neurodegeneration (Esposito et al., 2002). Thus, screening of compounds with an oxidant/antioxidant balancing property or a multitarget activity would offer an efficient neuroprotection.

A growing body of evidence demonstrates that varieties of herb materials exert their functions on the human body through a multitarget mechanism, i.e., an integrating, balancing, or coordinating action by corresponding active constituents (Sun et al., 2009; Wang et al., 2008). For instance, herbal food Purple *Perilla* fruit [the ripe fruit of *Perilla frutescens* (L.) Britt.] not only has antimicrobial (Yamamoto and Ogawa, 2002), antiallergic (Sanbongi et al., 2004; Takano et al., 2004), and antitumor (Osakabe et al., 2004; Ueda et al., 2003) efficacies, but also possesses immunomodulating (Ragažinskienė et al., 2004) and antioxidant (Korotkich et al., 2006; Meng et al., 2008) actions. From this herbal food, we previously isolated a polyphenolic compound luteolin (Zhao et al., 2009), which is actually a naturally-occurring compound also found in other foods including peanut shell, parsley, artichoke leaves, celery, peppers, olive oil, rosemary, lemons, peppermint, sage, thyme, and many others. This food-derived compound seems to be 1 of the dominant active constituents of Purple *Perilla* fruit because this isolate also possesses anti-inflammatory, antiallergic, anticarcinogenic, and immune-modulating properties (Van Zanden et al., 2004). Besides, this phytochemical with active polyphenolic hydroxyl groups can scavenge superoxide radicals such as hydroxyl radical, diphenylpicrylhydrazyl radical, and alkyl radical in cultured human endothelial cells (Choi et al., 2004) and hydrogen peroxide (H_2O_2) in a permanent immature rat oligodendrocytes cell line (van Meeteren et al., 2004) by preventing iron ion from binding to H_2O_2. Such ROS-scavenging property indicates, according to the role of ROS in neuronal apoptosis as mentioned above, that luteolin would possess a possible tendency to limit neurodegeneration in the central nervous system (CNS).

The objectives of our study were to establish stable in vitro drug screen models capable of mimicking neurodegeneration in humans, to screen potent neuroprotectants upon the platforms, and subsequently to elucidate the possible mechanisms of neuroprotection. Using primary cultured neurons and tumor cells of neural origin, various CNS-oriented phytochemicals with phenolic hydroxyl groups were subject to a screening program. Our primary screenings disclosed that luteolin was a potent neuroprotectant against ROS-induced neurotoxicity in the primary neurons but not in the human neuroblastoma cells (SK-N-SH).

2. Methods

2.1. Isolation of phytochemicals from herb source

Extraction, purification, and structural identification of luteolin from herb material Purple *Perilla* fruit [the ripe seed of *Perilla frutescens* (L.) Britt.] were conducted according to the methods reported previously (Zhao et al., 2009). Luteolin derivatives such as 5-isobutyryl luteolin, 5-acetyl luteolin, 7-propionyl luteolin, 5-octyl luteolin, and 7,3′,4′-tri-benzyl luteolin were kindly provided by Key Laboratory of Synthetic Chemistry of Natural Substances, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. Bakuchiol, Δ1,3-hydroxybakuchiol, and Δ3,2-hydroxybakuchiol used in this study were previously isolated from seeds of *Psoralea corylifolia* L. (Leguminosae) as reported by Zhao et al. (2008). Analogs of luteolin and bakuchiol were dissolved in double-distilled water containing 10% dimethylsulfoxide (DMSO; final concentration in reaction system, 1%) before experimentation.

2.2. Cell culture

Primary neurons were cultured as described previously (Singer et al., 1999) with slight modification. Cortices were harvested from brains of neonatal Sprague-Dawley rats at 12 hours of age, minced in dissociation buffer containing 0.14 M NaCl, 5.4 mM KCl, 24 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 4.2 mM $NaHCO_3$, 2.5 mM NaH_2PO4, and 14 mM glucose, and then treated with 0.25 mg/mL trypsin and 0.2 mg/mL deoxyribonuclease I followed by a 30-minute incubation at 37 °C. After inactivation of trypsin in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat-inactivated horse serum, cells were collected by centrifugation, seeded at concentration of 10^6 cells/mL on a poly-L-lysine-coated 6/96-well plate, and cultured in DMEM containing 10% heat-inactivated horse serum in an incubator (5% CO_2 in humidified chamber) at 37 °C. A B27-supplemented neurobasal culture medium (antioxidant-free) was substituted for DMEM before cell adhesion. Twenty-four hours later, cytarabine 10 μM was added to the medium for the purpose of inhibition of glial proliferation. Plates with enriched populations of neurons were adopted for experimentation. The primary cortical neurons were verified using an immunofluorescence technique. Cells were plated in a 6-well plate, washed 3 times with phosphate-buffered saline (PBS), and fixed in 2% paraformaldehyde for 15 minutes. The fixed cells were washed 3 times with PBS, permeabilized by treatment with 0.01% Triton x-100 in PBS at 37 °C for 15 minutes, and then blocked in PBS containing 50 mg/mL bovine serum albumin (BSA) for 2 hours. For immunofluorescence staining, cells were then incubated with antibody against microtubule associated protein 2 at a 1 : 1000 dilution before a 3-time PBS washing, and then incubated with a Cy3-labeled goat anti-rabbit IgG followed by another 3-time PBS washing (once every 10 minutes). For Hoechst staining, cells were incubated with Hoechst (1 : 1000) for 15 minutes under a light-free condition. The experiment was conducted in accord with accepted standards of humane animal care in accordance with the NIH Guide for the Care

and Use of Laboratory Animals and reviewed by the Laboratory Animal Center of the Chinese Academy of Science.

Human neuroblastoma SK-N-SH (Zhao et al., 2009) and human hepatoma HuH-7 cells (Alexia, 2004) were prepared as reported previously.

2.3. Cell survival measurements

The quantitation of cell mortality or viability upon treatment with H_2O_2 and/or luteolin was determined by the release of lactate dehydrogenase (LDH) from the cytosol of damaged cells using the cytotoxicity Detection Kit (Roche Diagnostics, Shanghai, China) as described previously (Riebeling et al., 2002), with a little modification. Briefly, primary neurons were culutured on a 96-cell plate for 4 days, then treated with test compounds or vehicle solvent, and then incubated for 12 hours. One hundred μL of culture medium was then transferred to another 96-cell plate, into which 100-μL reaction mixture (containing 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyl tetrazolium chloride, diaphorase, nicotinamide adenine dinucleotide, and sodium lactate) was added. After a 30-minute incubation at 37 °C (protected from light), the extinction at 490 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader. The extinction values of control wells (where cells were lysed in 1% Triton x-100 solution 30 minutes before transferring supernatants) were set as 100% and the rate of LDH release from treated cells was calculated as percent of control (background was recouped). The survival rate was quantified as the percentage of live cells (100% minus death rate). Because the product of the biochemical reaction was red, the cells were allowed to be incubated in a phenol red-free DMEM supplemented with 10% fetal bovine serum to avoid colorimetric interference.

For assessing whether luteolin itself could affect cell viability, a highly sensitive 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) reduction was assayed upon SK-N–SH, HuH-7, and primary cultured neurons. After a 12-hour reatment with luteolin, the culture was administered with WST-8 agent and incubated for 3 additional hours at 37 °C before a colorimetry [conducted according to the manufacturer instructions of the Cell Counting Kit 8 (CCK-8) (Dojindo Laboratories, Tokyo, Japan)]. WST-8 reduction in each group was expressed as the optical density (OD) ratio between the luteolin-treated cell group and luteolin concentration-paired, cell-free group. Such normalization is used for recouping the interference produced by the light yellow color of luteolin solvent.

2.4. Assays of mitochondrial viability and mitochondrial membrane potential

Mitochondrial activity was assayed by 3-(4,5-dimethylthiazohl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, St. Louis, MO) reduction, in which the mitochondrial marker succinate dehydrogenase can reduce its yellow substrate MTT into violet substance formazan. Upon experimentation, primary cultured cortical neurons were incubated with H_2O_2 and/or differing concentrations of luteolin for 12 hours at 37 °C. MTT reagent (with a final concentration of 0.5 mg/mL) was then added to the system, and the dyeing reaction persisted for 3 hours until purple precipitate was visible. The supernatant was removed, and a decolorizing solution (Triton x-100: dimethylformamide: H_2O = 1:3:2, in 0.2 M citric acid solvent; pH 5-6) was added, followed by a 30-minute incubation. The absorbance of reaction solution at 570 nm was measured. Blank control was performed by obviating cells in the wells. Mitochondrial index was rated by the OD ratio between the indicated compound- and vehicle-treated cell groups.

Changes in mitochondrial membrane potential ($\Delta\Psi$m) were measured by utilizing potential-sensitive substance rhodamine 123 (Sigma) as previously described (Wu et al., 2006). Briefly, primary neurons were incubated (15 minutes, 37 °C, in the dark) in the culture medium containing 100 nM rhodamine 123. Data were analyzed by FACS Calibur with CellQuest software version 5.1 (B&D, San Jose, CA).

2.5. Determination of free radical production

Free radical production was measured by incubation of the primary neurons with the fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma) (Tammariello et al., 2000). DCFH-DA freely crosses cell membranes and it is hydrolyzed by cellular esterases into 2′,7′-dichlorodihydrofluorescein ($DCFH_2$), a nonfluorescent molecule that can be oxidized into fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of peroxides. Ten micromolar probe DCFH-DA was added into the drug-treated cell wells, and following a 30-minute incubation at room temperature, DCF accumulation in the cells was measured as an increase of fluorescence at 525 nm when excited at 488 nm using a spectrofluorimeter.

2.6. Assays of catalase and glutathione

Primary neurons were collected from the 6-cell plate by scraping. PBS washing was followed by an addition of cell immunoprecipitation lysis buffer or detergent M solvent to the reaction system. Catalase activity and glutathione level were then assayed by the methods of He et al. (2008) and Bi et al. (2008), respectively. Both assay kits were purchased from Beyotime Institute of Biotechnology, Shanghai. Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as standard.

2.7. Data analysis

Data were analyzed using SPSS software v. 13.0 (Chicago, IL). Values were expressed as mean ± standard error of the mean (SEM). One-way/2-way analysis of variance (ANOVA) followed by post hoc tests with least significant difference (LSD) (under variance homogeneity) or with

Tamhane's T_2 (under variance heterogeneity) was used to examine differences between groups, with the exception that comparison of the negative logarithm of the drug concentration eliciting half maximal response (pEC_{50}) and the maximal response (E_{max}) between 2 groups was analyzed by a Student independent t test. pEC_{50} and E_{max} were generated by GraphPad Prism v. 5.0 (GraphPad Software, San Diego, CA), in which the curve-fitting equation for sigmoidal dose-response was:

$$Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{(LogEC_{50} - X) \times HillSlope}}.$$

For all comparisons, $p < 0.05$ was considered statistically significant.

3. Results

3.1. Verification of the in vitro screen platforms

Primary neurons from newborn rat cortex attached to polyvinyl chloride plates 3 hours after seeding, and neurites began to outgrow 24 hours later. As shown in Fig. 1a, Hoechst/microtubule associated protein 2 staining was clear under a Leica immunofluorescence microscope (Leica, Bernstein, Germany), demonstrating a perfect viability and a CNS-derived neuronal property. When the primary neurons or SK-N-SH cells were insulted with differential concentrations of H_2O_2 followed by a 12-hour incubation, cell mortality significantly decreased in a concentration-dependent manner (primary neurons/SK-N-SH, 2-way ANOVA $F(5,12) = 236.22/233.15$, $p < 0.001$; posthoc $p = 0.11/0.76$ or $p = 0.001/0.086$ respectively for 10 μM or 50 μM H_2O_2

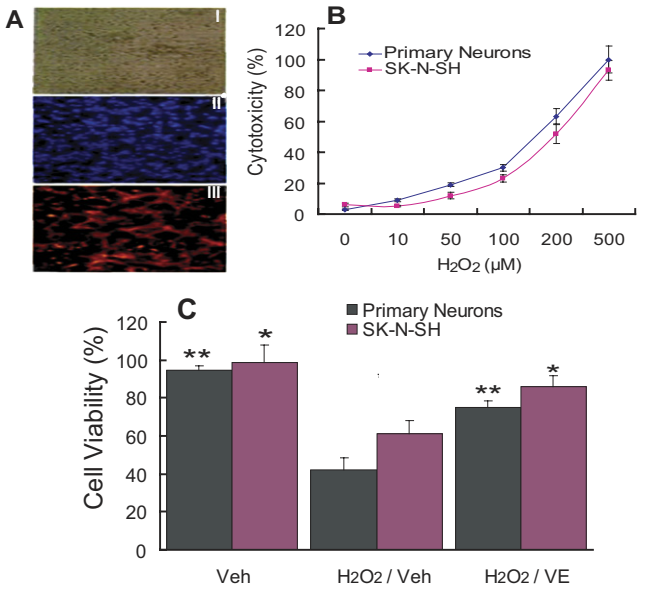

Fig. 1. Quality control for the in vitro screen systems. (a) Morphological identification of primary culture neurons, wherein I, II, and III represent cells with nonstaining, Hoechst staining, and microtubule associated protein 2 staining, respectively (×400). (b) Effects of differential concentrations of H_2O_2 on cell viability in primary neurons and human neuroblastoma SK-N-SH cells assayed by lactate dehydrogenase (LDH)-releasing test. (c) Platform validation by reference vitamin E (VE). Cells were treated with 200 μM H_2O_2 together with 50 μM VE and cell viability was assayed by LDH test. * and **, $p < 0.005$ and 0.001, respectively, compared with cells with H_2O_2/vehicle (Veh) by 1-way analysis of variance (ANOVA) followed by posthoc least significant difference (LSD). Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples.

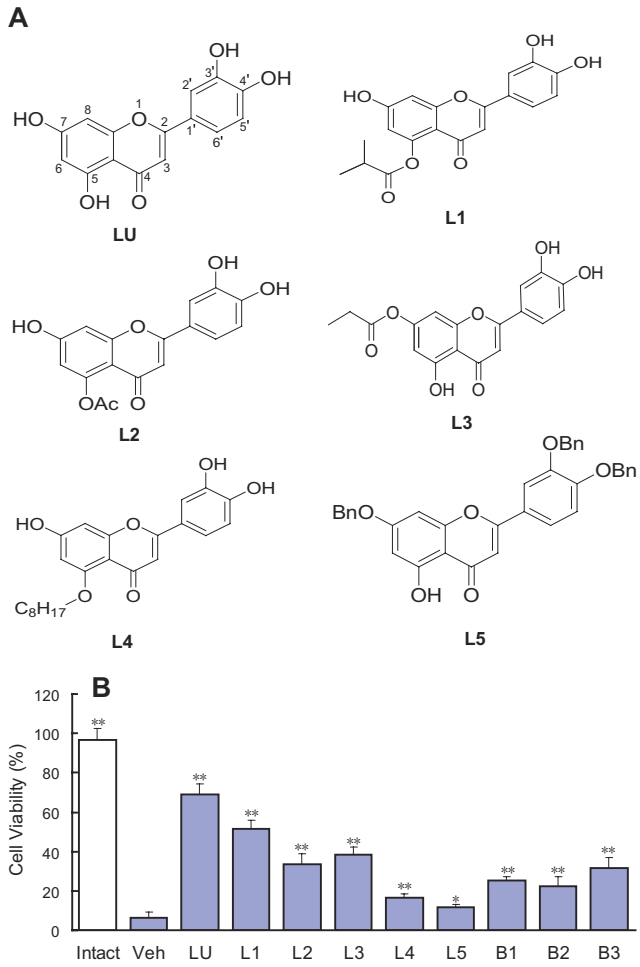

Fig. 2. Primary screening of neuroprotective agents. (a) Molecular structure of polyphenolic compounds used for primary screenin: LU, luteolin; L1, 5-isobutyryl luteolin; L2, 5-acetyl luteolin; L3, 7-propionyl luteolin; L4, 5-octyl luteolin; and L5, 7,3′,4′-tri-benzyl luteolin. (b) Effect of polyphenolic compounds on the viability of H_2O_2-insulted primary neurons (by lactate dehydrogenase [LDH] assay): luteolin (LU), 5-isobutyryl luteolin (L1), 5-acetyl luteolin (L2), 7-propionyl luteolin (L3), 5-octyl luteolin (L4), 7,3',4'-tri-benzyl luteolin (L5), bakuchiol (B1), Δ1,3-hydroxybakuchiol (B2), and Δ3,2-hydroxybakuchiol (B3) each at concentration of 10 μM were used to treat the H_2O_2 (200 μM)-insulted neurons (solid bars). Intact, cell wells without treatment (blank bar). The compounds themselves did not affect the viability of primary neurons (data not shown). * and **, $p < 0.05$ and 0.01, respectively, compared with vehicle (Veh) 1 one-way analysis of variance (ANOVA) followed by posthoc least significant difference (LSD). Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples.

vs. intact cells, and all $p < 0.001/< 0.001$ for 100, 200, and 500 μM H_2O_2 vs. intact cells), wherein H_2O_2 at concentrations up to 500 μM induced around 100% mortality (Fig. 1b). Following a 12-hour concomitant treatment with VE (final concentration 50 μM in reaction system containing 1% dimethylsulfoxide) and H_2O_2 (200 μM), the 2 types of insulted cells exhibited higher levels of viability relative to H_2O_2 only-treated cells (primary neurons/SK-N-SH, 1-way ANOVA $F(2,6) = 119.14/20.95$, $p < 0.001/= 0.02$; LSD $p < 0.001/= 0.006$ vs. insulted control cells) (Fig. 1c), demonstrating a neuroprotective action produced by this reference as well as confirming the validity and feasibility of the in vitro models for screening neuroprotectants.

3.2. Primary screening and effect of luteolin on H_2O_2-insulted primary neurons

Using LDH assay upon the 2 screens, we primarily screened neuroprotective agents from a series of CNS-oriented compounds. Our initial results showed that several polyphenolic compounds, such as luteolin,analogs (luteolin and its 5-isobutyryl, 5-acetyl, 7-propionyl, 5-octyl, and 7,3′,4′-tri-benzyl substitute) and bakuchiol analogs (bakuchiol and its Δ^1,3-hydroxy and Δ^3,2-hydroxy substitute) (Fig. 2a), each at concentrations of 10 μM, significantly rescued primary neurons from H_2O_2 insult (1-way ANOVA $F(10,22) = 459.14$, $p < 0.001$); the degrees of their activities were different, wherein luteolin, an isolate of Purple *Perilla* fruit, is with a preferential action for enhancing the viability of ROS-intoxicated neurons (Fig. 2b), which was therefore adopted as a candidate for further study. The general rank order for the actions of luteolin analogs was luteolin > 5-isobutyryl-luteolin > 5-acetyl-luteolin ≈ 7-propionyl-luteolin > 5-octyl-luteolin ≈ 7,3′,4′-tri-benzyl-luteolin (Fig. 2b).

To explore whether the indicated bioactivity is different between differing screen platforms, both primary neurons and SK-N-SH cells were used and treated with luteolin and stressed with H_2O_2 (200 μM) and LDH release was then assayed. A 2-way ANOVA analysis of the viability of primary neurons revealed a significant interaction between luteolin administration and H_2O_2 insult (luteolin × H_2O_2, $F(3,16) = 14.7$, $p < 0.001$), and significant main effects of luteolin ($F(3,16) = 10.68$, $p < 0.001$) and H_2O_2 insult ($F(1,16) = 96.13$, $p < 0.001$); LSD multiple comparison revealed significant differences between vehicle and luteolin (at arbitrary concentrations of 5, 10, and 20 μM) ($p = 0.045/= 0.001/< 0.001$, respectively), demonstrating a protective action on the viability of H_2O_2-insulted primary neurons. However, a different thing was shown in SK-N-SK cells after 2-way ANOVA analysis, which disclosed that there was no significant interaction in the cell viability between luteolin administration and H_2O_2 insult (luteolin × H_2O_2, $F(3,16) = 1.71$, $p = 0.21$), while there was significant main effects of luteolin ($F(3,16) = 9.96$, $p = 0.001$) and H_2O_2 insult ($F(1,16) = 409$, $p < 0.001$); LSD multiple comparison revealed significant differences between vehicle and luteolin (for 10 μM or 20 μM group, $p = 0.008/< 0.001$, respectively) (Fig. 3a), reflecting that luteolin provoked a worsening of H_2O_2 cytotoxicity. Twenty micromolar luteolin alone seemed to decrease the proliferation of this tumor cell in the absence of H_2O_2 but with no statistical significance when comparing to the intact cells (Fig. 3a). To test this possible antitumor action, luteolin itself within a wider concentration range was added into the neuroblastoma SK-N-SH cells or reference HuH-7 cell line and primary neurons, and then their viabilities were assayed by using a sensitive WST-8 test. Results showed that luteolin mildly inhibited the viability of SK-N-SH cells (50% inhibition at 68.7 μM) and relatively strongly inhibited that of HuH-7 cells (50% inhibition at 14.3 μM), but did not affect that of primary neurons (Fig. 3b). Thus, luteolin prevented against ROS-induced neural cytotoxicity selectively in the primary neurons, which then served as an appropriate platform for the following pharmacological study.

For corroborating the preliminary concept of neuroprotection and for comparing the effectiveness of indicated

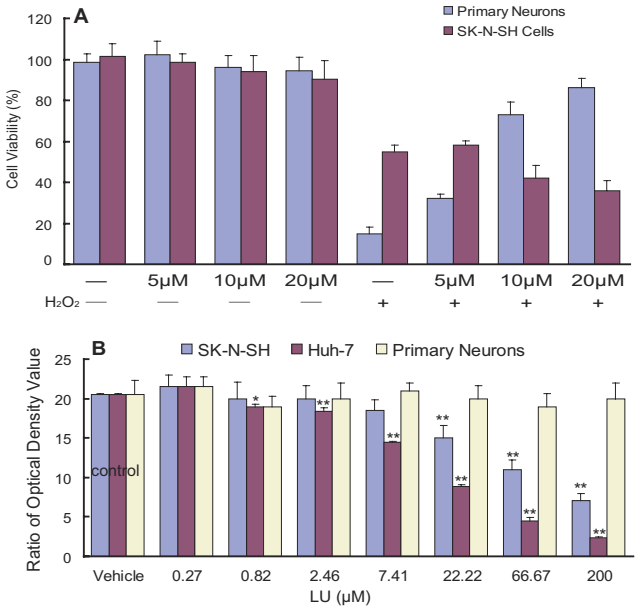

Fig. 3. Modulating effects of luteolin (LU) on the viability of H_2O_2-insulted or noninsulted cells of neural origin. (a) A primary evaluation of the modulating actions of LU (5, 10, and 20 μM) (lactate dehydrogenase [LDH] assay) on the viability of H_2O_2 (200 μM)-insulted primary neurons/human neuroblastoma SK-N-SH cells (analyzed by 2-way analysis of variance [ANOVA]) followed by posthoc least significant difference [LSD]). LU significantly enhanced the survival of H_2O_2-insulted primary neurons in a concentration-dependent manner, but exacerbated the H_2O_2-induced damage in SK-N-SH cells. (b) Effects of different concentrations of LU on cell viability in SK-N-SH, human hepatoma HuH-7 cells, and primary neurons (H_2O_2-free): assayed by Cell Counting Kit 8 (CCK-8) test (Dojindo Laboratories, Tokyo, Japan). * and **, $p < 0.005$ and 0.001, respectively, compared with respective vehicle-treated control cells by 1-way ANOVA followed by posthoc LSD. Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples.

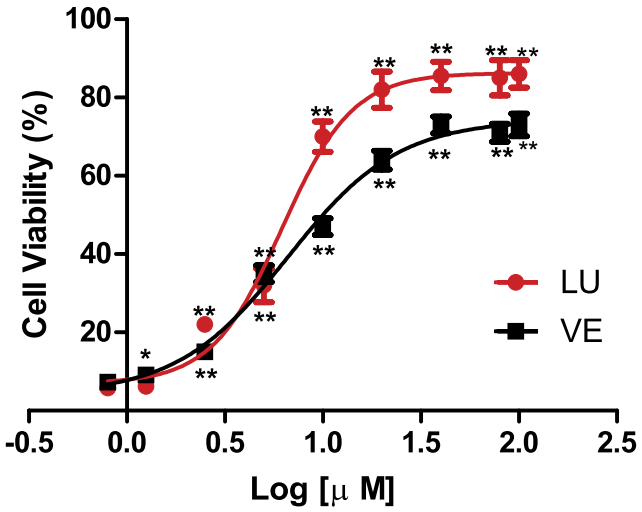

Fig. 4. Concentration-response curve for neuroprotective actions of luteolin (LU) and vitamin E (VE) in H_2O_2 (200 μM)-insulted primary neurons (lactate dehydrogenase [LDH] test). Optical density value of vehicle control, 5.3 ± 0.81 or 5.71 ± 0.92 respectively for the reaction system with LU or VE. One-way analysis of variance (ANOVA) for LU/VE, $F(9,20) = 375.09/F(9,20) = 648.32$, $p < 0.001/p < 0.001$, respectively. Both compounds at concentrations 0.8 μM to 100 μM produced concentration-dependent responses with S-type curves, and the negative logarithm of the drug concentration eliciting half maximal protection and the maximal protection (summarized in Table 1) were generated following a curve-fitting algorithm (each $R^2 \approx 0.99$, Hill slope = 1 ~ 1.7). * and **, $p = 0.049$ and $p < 0.001$, respectively, compared with insulted control cells (posthoc least significant difference [LSD]). Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples.

compounds, luteolin or VE, each in a broader concentration range (0.8–100 μM), was added to the primary neurons. After 12-hour coincubation with H_2O_2 (200 μM), LDH release was detected. Semilogarithmic concentration-effect curves were established, and then the parameters pEC_{50} and E_{max} were generated using GraphPad. As shown in Fig. 4, each compound engendered concentration-dependent actions and generally shaped S-type curves. pEC_{50} and E_{max} (expressed as survival rate) for enhancing cell viability are given in Table 1. The 2 compounds exhibited different pEC_{50} and E_{max} values, wherein the 2 values for luteolin were mildly higher than that of VE, with significance for

Table 1
pEC_{50} and E_{max} of luteolin (LU) and vitamin E (VE) for upregulating survival rate of H_2O_2-untoxified primary neurons

	pEC_{50} [a]	E_{max} [b]
LU	−0.79 ± 0.15	86.21 ± 6.23
VE	−1.12 ± 0.11	72.27 ± 5.14

Values are the means ± standard error of the mean (SEM) of 3 independent experiments performed in triplicate. pEC_{50}/E_{max}, $p = 0.067/0.048$, compared with group with VE (2-tailed independent samples t test).

[a] Negative logarithm of the drug concentration eliciting half maximal protection (pEC_{50}): −log [μM].

[b] Maximal protection (E_{max}): %.

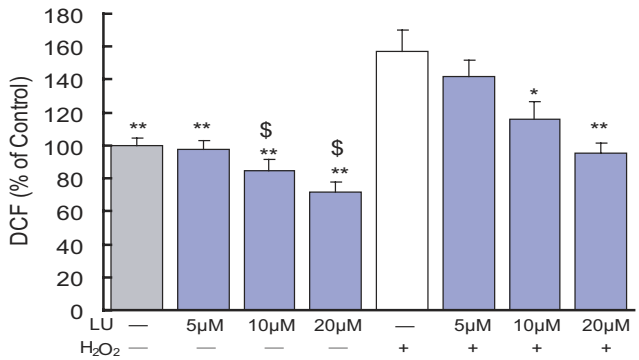

Fig. 5. Effect of luteolin (LU) on reactive oxygen species (ROS) production in the H_2O_2-insulted primary neurons. LU itself (at 5, 10, and 20 μM) mildly decreased fluorescent 2′,7′-dichlorofluorescein (DCF) level while H_2O_2 (200 μM) significantly increased DCF production following a 12-hour incubation of primary neurons. LU at the indicated concentrations markedly reversed the increase of DCF level in the insulted neurons. Cell group of nontreatment was set as 100%. $, $p < 0.05$, compared with nontreatment group (gray bar); * and **, $p < 0.05$ and 0.001, respectively, compared with insulted control cells (open bar). Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples and analyzed by 2-way analysis of variance (ANOVA) followed by posthoc least significant difference (LSD).

luteolin E_{max} ($p = 0.048$ by Student t test) but with no significance for luteolin pEC_{50} ($p = 0.067$).

3.3. Effect of luteolin on ROS content in the injured neurons

Intracellular production of ROS was detected by using the ROS indicator DCF as a probe. A 2-way ANOVA analysis of DCF production on primary culture neurons revealed a significant interaction between luteolin administration and H_2O_2 insult (luteolin × H_2O_2, $F(3,16) = 5.07$, $p = 0.011$) as well as significant main effects of luteolin ($F(3,16) = 37.93$, $p < 0.001$) and H_2O_2 insult ($F(1,16) = 143.75$, $p < 0.001$). The DCF-fluorescence in the primary neurons was substantially increased when the cells were insulted by H_2O_2 (posthoc LSD, $p < 0.001$ vs. intact group). By contrast, treatment with luteolin itself produced a mild inhibitory action on the ROS production in the primary neurons with statistical significance for 20 μM and 40 μM concentration groups (posthoc LSD, $p = 0.037$ and 0.01 respectively, vs. intact group). Upon the H_2O_2-insulted cells, luteolin strikingly improved the abnormal increase of intracellular DCF level (posthoc LSD, $p = 0.03$, $p < 0.001$, or $p < 0.001$, respectively for 5, 20, or 40 μM group vs. H_2O_2 only-treated cells) (Fig. 5).

3.4. Effect of luteolin on the mitochondrial function in ROS-injured neurons

Formazan produced in neuronal mitochondria was assayed with MTT. A 2-way ANOVA analysis of the formazan production revealed a significant interaction between luteolin administration and H_2O_2 insult (luteolin × H_2O_2,

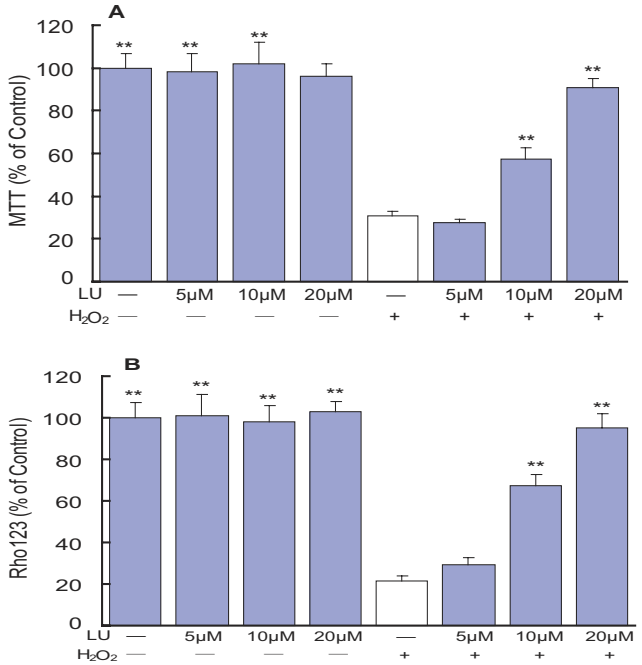

Fig. 6. Effect of luteolin (LU) on the mitochondrial functions in the H_2O_2-insulted primary neurons. LU itself (at 5, 10, and 20 μM) did not affect mitochondrial functions ($p > 0.05$ vs. intact cells) while H_2O_2 (200 μM) significantly inhibited mitochondrial functions following a 12-hour incubation of primary neurons. LU at 10 μM and 50 μM markedly reversed the decrease in formazan level (a) in 3-(4,5-dimethyl-thiazohl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and the depression in mitochondrial potential (b) in the insulted neurons in rhodamine 123 (Rho123) assay. Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples. **, $p < 0.001$, compared with insulted cell control group (open bar) by 2-way analysis of variance (ANOVA) followed by posthoc least significant difference (LSD).

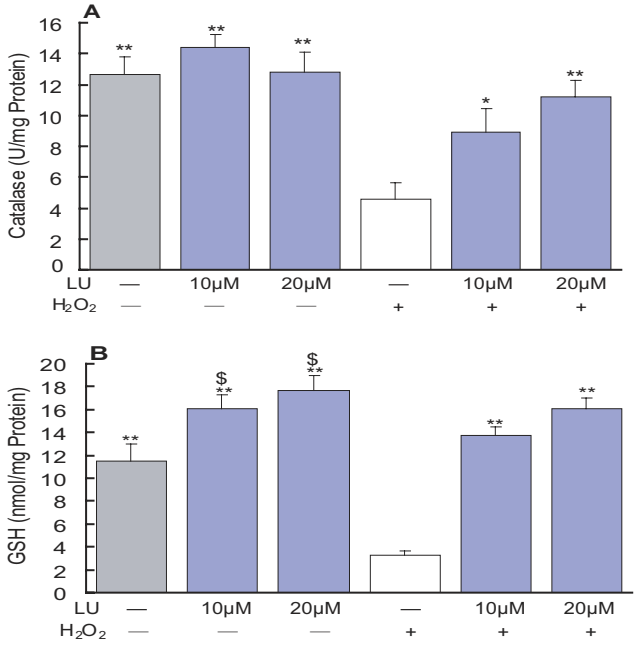

Fig. 7. Effects of luteolin (LU) on catalase activity and glutathione level in H_2O_2-insulted primary neurons. Following a 12-hour incubation of primary neurons, LU itself (at 10 μM and 20 μM) did not affect catalase activity (a) but did increase glutathione (GSH) level (b), while H_2O_2 (200 μM) decreased the levels of both endogenous enzymatic and nonenzymatic antioxidants. LU markedly reversed the decrease of catalase activity (a) and GSH level (b) in the insulted neurons. * and **, $p < 0.005$ and 0.001, respectively, compared with insulted cells (open bar); $, $p < 0.05$, compared with nontreatment group (gray bar). Values are expressed as mean ± standard error of the mean (SEM) of triplicate cell samples and analyzed by 2-way analysis of variance (ANOVA) followed by posthoc least significant difference (LSD).

$F(3,16) = 36.74$, $p < 0.001$) as well as significant main effects of luteolin ($F(3,16) = 35.65$, $p < 0.001$) and H_2O_2 insult ($F(1,16) = 352.01$, $p < 0.001$). As shown in Fig. 6a, the levels of formazan in luteolin only-treated cells (5, 10, and 20 μM) were not different from intact cells, while that in H_2O_2 only-treated cells was markedly lower than that in intact cells (posthoc $p < 0.001$). However, the decrease of formazan production in H_2O_2-insulted cells was strikingly reversed by luteolin with significance for 10 μM and 20 μM luteolin (both posthoc $p < 0.001$ vs. H_2O_2 only-treated cells).

For further confirming the protective action in mitochondria, another functional marker mitochondrial membrane potential ($\Delta\Psi$m) was detected on the primary neurons, into which the toxin H_2O_2 together with luteolin was added. A 12-hour incubation showed a significant interaction in membrane potential between luteolin administration and H_2O_2 insult (2-way ANOVA, luteolin × H_2O_2, $F(3,16) = 40.22$, $p < 0.001$) as well as significant main effects of luteolin ($F(3,16) = 43.69$, $p < 0.001$) and H_2O_2 insult ($F(1,16) = 315$, $p < 0.001$). As shown in Fig. 6b, the membrane potential in neuronal cells (H_2O_2-free) treated with luteolin was generally similar to that in intact cells, while that in H_2O_2 only-treated cells was lower than that in intact cells (posthoc LSD $p < 0.001$). When the insulted cells were treated with luteolin, a concentration-dependent enhancing effect on membrane potential was produced (for 10 and 20 μM luteolin, both $p < 0.01$ vs. H_2O_2 only-treated group).

3.5. Effects of luteolin on catalase and glutathione in ROS-injured neurons

Actions of luteolin on catalase (Fig. 7a) and glutathione (Fig. 7b) were explored on primary neurons. A 2-way ANOVA analysis of catalase/glutathione activity revealed a significant interaction between luteolin administration and H_2O_2 insult (catalase/glutathione: luteolin × H_2O_2, $F(2,12) = 11.17/16.48$, $p = 0.002/< 0.001$) as well as significant main effects of luteolin (catalase/glutathione: $F(2,12) = 15.08/126.84$, $p = 0.001/< 0.001$) and H_2O_2 insult (catalase/glutathione: $F(1,12) = 81.91/62.18$, $p < 0.001/< 0.001$). In the H_2O_2-free neurons, luteolin at indicated concentrations did not affect the activity of catalase while at several concentration points did significantly increase the amount of glutathione. Compared with intact

neurons, catalase/glutathione amounts in the H_2O_2-insulted neurons was significantly decreased (both $p < 0.01$). However, the decreases of catalase/glutathione activity by H_2O_2 were markedly reversed following luteolin treatment. Detailed multiple comparisons are shown in the legend for Fig. 7.

4. Discussion

ROS, such as partially reduced oxygen species including superoxide anion ($O2^{\cdot -}$), H_2O_2, and hydroxyl radical ($OH^{\cdot}$) (Barinaga, 1998), are mainly generated endogenously as byproducts of respiration in mitochondria. Under normal metabolic conditions, the mitochondria-generated ROS are rapidly scavenged by various enzymatic and nonenzymatic antioxidants, leading to a maintenance of balance between oxidative attack and antioxidant defense systems prevailing in different tissues (Kehrer, 1993). Nonetheless, the brain has a weak capacity for antioxidative defense (Kim et al., 2000) and thereby is oxidatively sensitive and vulnerable (Lewen et al., 2000). Under certain pathological conditions, catastrophically increased intracellular ROS are formed, which attach to membrane polyunsaturated fatty acids and proteins, thereby inflicting lipid peroxidation and phosphatidylcholine and also increasing membrane permeability (López et al., 2006). Thus, the increased oxidative stress in cerebral tissue is 1 of the major contributing factors for the development of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and cerebral ischemia-reperfusion in aged individuals (Casetta et al., 2005; Li et al., 2005). According to this notion, neurodegenerative diseases may be plausibly modeled in vitro by administration of exogenous ROS to neural cell lines or primary cultured neurons; and by doing so, cell platforms capable of screening clinically meaningful neuroprotectants could be established.

Rat primary cultured neurons (Fig. 1a) and human neuroblastoma SK-N-SH cells seemed to meet our current demands because viabilities of the 2 types of cells were able to be strikingly inhibited when exposed to exogenous ROS (Fig. 1b). For verification of the in vitro screen platforms, the 2 cell systems were treated with a known potent antioxidant VE immediately before addition of H_2O_2. The results showed that this reference compound significantly reversed the H_2O_2-induced damage in the 2 platforms (Fig. 1c). Replicate variability was less than 10%, confirming their validity and feasibility for drug screening.

H_2O_2, a classic oxidative stressor that is the dismutation product of superoxide and a precursor to hydroxyl radicals, has been widely utilized as a tool for developing cell damage models (Jiang et al., 2001). As shown in LDH assay, a cytotoxic phenomenon took place after cultured neural cells were insulted with this highly toxic compound (Figs. 1b, 2b, and 3a). It is well known that high levels of H_2O_2 attack mitochondrial respiratory chains, not only resulting in an energy deficiency either by inhibition of mitochondrial membrane potential or by enhancement of membrane permeation but also contributing to a subsequent increase of ROS production and decrease of endogenous antioxidant content (Kovacic et al., 2005). Thus, the cytotoxicity at the cellular level in our insulted primary neurons was accompanied by a series of damaging events not only at the subcellular level such as the depression of mitochondrial viability (Fig. 6a) and decrease of mitochondrial membrane potential (Fig. 6b), but also at molecular level such as the increase of intracellular ROS (Fig. 5) and decreases of glutathione level and catalase activity (Fig. 7), all of which simultaneously occurred and may account for a secondary imbalance between the oxidant and antioxidant networks likely involved in a proapoptotic cascade (Simon et al., 2000). As indicated by the phenomenon of decrease in formazan OD value (Fig. 6a), the proapoptotic event may occur following oxidative insult in the primary neurons.

Upon the platforms, our primary investigation revealed that luteolin presented a preferential neuroprotective action among candidate phytochemicals (Fig. 2b) and its protection was toward the primary neurons but not to SK-N-SH cells (Fig. 3a). Thus, luteolin was chosen as a candidate for further evaluation while the primary neurons served as its optimal evaluation platform. Also, our primary screening showed that the degree of protective action by luteolin decreased following isobutyryl substitution of hydroxyl group at the C-5 position of luteolin, further decreased following 5-acetyl or 7-propionyl substitution, and even further decreased after 5-octyl or 7,3′,4′-tri-benzyl substitution (Fig. 2b), demonstrating key roles of hydroxyl and long chain alkyl groups in the structure-activity relationships.

For defining the efficacy of luteolin and its potential for application, a reference compound is needed. VE may reduce the recurrence of stroke for patients experiencing high oxidative stress (Kidd, 2009), and treatment of primary hippocampal neurons or *Oxys* rats with VE protects against oxidative damage (de Jesus Ferreira et al., 2005; Kolosova et al., 2006); VE was thus chosen as a reference for efficacy comparison. After incubation of the primary neurons with a wider range of concentrations of luteolin or reference, our results showed an excellent ability of luteolin to protect neurons against ROS attack with an efficacy (maximal cell survival) higher than and a potency comparable to VE (Fig. 4, Table 1), demonstrating that luteolin can be designated as a potent neuroprotectant as well as suggesting that it may be effective either in the treatment of neurodegenerative diseases, such as cerebral ischemia, Parkinson's disease, and Alzheimer's disease, or in the improvement of brain aging. Besides, luteolin also produced counteractive effects on oxidative insult-associated downstream events such as the abnormal increase of endogenous free radicals (Fig. 5), inhibition of mitochondrial viability (Fig. 6a), loss of mitochondrial membrane potential (Fig. 6b), and decreases of

glutathione content and catalase activity (Fig. 7), indicating that multilayer modulatory pathways may participate in the luteolin's neuroprotection and that this protection may be partially due to a possible balancing of pro-oxidant/antioxidant ratio. On the 1 hand, the improvement of mitochondrial viability and enhancement of membrane potential in the damaged neurons may possibly contribute to an antiapoptotic action by reduction of cytochrome c release from mitochondria into cytosome, a process that is known to be associated with activation of proapoptotic factor caspase-9 (Wu et al., 2006). By contrast, enhancements of glutathione level and catalase activity indicate that luteolin acts by neuroprotection possibly through mechanisms of restoration of the depressed endogenous enzymatic and nonenzymatic antioxidant defense systems besides its known ROS scavenging activity (Choi et al., 2004).

Additionally, as mentioned above, the neuroprotective action of luteolin was exclusively upon the ROS-insulted primary neurons because there was, unexpectedly, no protecting effect seen in the ROS-insulted neuroblastoma SK-N-SH cells. This dichotomy between the 2 platforms may be due to triggering of the signaling pathways involved in differing procecess by luteolin and resulting either in a subsequent antioxidation or in antitumor; but their definite mechanisms need to be studied in the near future. As shown in Fig. 3b, luteolin itself possesses a strong inhibitory effect on the viability of HuH-7 cells, consistent with a report that luteolin has an anticarcinogenic action on the human mammary carcinoma line MCF-7 (Van Zanden et al., 2004); while it also inhibited that of neuroblastoma SK-N-SH cells though with a potency weaker than that on HuH-7 cells, strongly supporting our antitumor supposition in the SK-N-SH platform. Besides, previous study by others showed that cellular endogenous H_2O_2 played a role in the apoptosis of epithelial Hela cells (Cai et al., 2008); and our current study revealed that, on the SK-N-SH cells instead of primary culture neurons, there was no significant interaction in cell viability between luteolin administration and ROS insult (Fig. 3a). Accordingly, it is further suggested that combination of luteolin with H_2O_2 toward the neuroblastoma cells would result in an obvious antitumor action instead of antioxidation, a supposition that may be appropriate to explain the aggravation of H_2O_2-induced cytotoxicity by luteolin in SK-N-SH cells (Fig. 3a) (possibly via a mitochondria-dependent apoptosis pathway as indicated by the WST-8 agent in Fig. 3b). Moreover, the fact that the viability of primary neurons was not affected by luteolin (Fig. 3b, and Fig. 6) indicates that this established in vitro model is more suitable for the screening of neuroprotectants from particular candidate compounds with a known antitumor property or an antitumor potential. We also indicate that utilization of nonneoplastic neural cells may escape from potential false negatives during screening of neuroprotectants. The results from our current study will point out a direction for future in vitro screening of neuroprotective pharmaceuticals.

In summary, the food-derived constituent luteolin functions as neuroprotective selectively in primary cultured neurons exposed to oxidative stress, an action that appears to be partially through a rebalancing of the aberrated oxidant/antioxidant status. The neuroprotectant luteolin as a dietary supplement or a pharmaceutical may be effective in the treatment of neurodegenerative diseases such as cerebral ischemia, Parkinson's disease, and Alzheimer's disease as well as in improvement of brain aging possibly through a multitargeting mechanism, because luteolin also has anti-inflammatory, immunomodulating (Theoharides, 2009), and anxiolytic-like (Coleta and Campos, 2008), memory-acquisition ameliorating (Tsai et al., 2007) actions as well as an inhibitory effect on β-site amyloid precursor protein-cleaving enzyme-1 (Choi et al., 2008) in addition to antioxidant action, each of which is known to be an alternative approach to the treatment of neurodegenerative conditions.

Disclosure statement

There are no actual or potential conflicts of interest, including any financial, personal, or other relationships with people or organizations that could inappropriately influence the current study.

This study was conducted in accord with accepted standards of humane animal care in accordance with the NIH Guide for the Care and Use of Laboratory Animals and reviewed by the Laboratory Animal Center of the Chinese Academy of Science.

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References

Alexia, C., Fallot, G., Lasfer, M., Schweizer-Groyer, G., Groyer, A., 2004. An evaluation of the role of insulin-like growth factors (IGF) and of type-I IGF receptor signalling in hepatocarcinogenesis and in the resistance of hepatocarcinoma cells against drug-induced apoptosis. Biochem. Pharmacol. 68, 1003–1015.

Barinaga, M., 1998. Stroke damaged neurons may commit cellular suicide. Science 281, 1302–1303.

Bi, J., Jiang, B., Liu, J.H., Zhang, X.L., An, L.J., 2008. Protective effects of catalpol against H(2)O(2)-induced oxidative stress in astrocytes primary cultures. Neurosci. Lett. 442, 224–227.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cai, Y.J., Lu, J.J., Zhu, H., Xie, H., Huang, M., Lin, L.P., Zhang, X.W., Ding, J., 2008. Salvicine triggers DNA double-strand breaks and ap-

optosis by GSH-depletion-driven H_2O_2 generation and topoisomerase II inhibition. Free Radic. Biol. Med. 45, 627–635.

Calabrese, V., Bates, T.E., Stella, A.M., 2000. NO synthase and NO-dependent signal pathways in brain aging and neurodegenerative disorders: the role of oxidant/antioxidant balance. Neurochem. Res. 25, 1315–1341.

Calabrese, V., Colombrita, C., Sultana, R., Scapagnini, G., Calvani, M., Butterfield, D.A., Stella, A.M., 2006. Redox modulation of heat shock protein expression by acetylcarnitine in aging brain: relationship to antioxidant status and mitochondrial function. Antioxid. Redox Signal. 8, 404–416.

Casetta, I., Govoni, V., Granieri, E., 2005. Oxidative stress, antioxidants and neurodegenerative diseases. Curr. Pharm. Des. 11, 2033–2052.

Choi, J.S., Choi, Y.J., Park, S.H., Kang, J.S., Kang, Y.H., 2004. Flavones mitigate tumor necrosis factor-alpha-induced adhesion molecule up-regulation in cultured human endothelial cells: role of nuclear factor-kB. J. Nutr. 134, 1013–1019.

Choi, S.H., Hur, J.M., Yang, E.J., Jun, M., Park, H.J., Lee, K.B., Moon, E., Song. K.S., 2008. Beta-secretase (BACE1) inhibitors from Perilla frutescens var. acuta. Arch. Pharm. Res. 31, 183–187.

de Jesus Ferreira, M.C., Crouzin, N., Barbanel, G., Cohen-Solal, C., Récasens, M., Vignes, M., Guiramand, J., 2005. A transient treatment of hippocampal neurons with alpha-tocopherol induces a long-lasting protection against oxidative damage via a genomic action. Free Radic. Biol. Med. 39, 1009–1020.

Coleta, M., Campos M.G., Cotrim, M.D., Lima, T.C., Cunha, A.P., 2008. Assessment of luteolin (3′,4′,5,7-tetrahydroxyflavone) neuropharmacological activity. Behav. Brain Res. 189, 75–82.

Esposito, E., Rotilio, D., Di Matteo, V., Di Giulio, C., Cacchio, M., Algeri, S., 2002. A review of specific dietary antioxidants and the effects on biochemical mechanisms related to neurodegenerative processes. Neurobiol. Aging 23, 719–735.

He, Z., Sun, X., Mei, G., Yu, S., Li, N., 2008. Nonclassical secretion of human catalase on the surface of CHO cells is more efficient than classical secretion. Cell Biol. Int. 32, 367–373.

Hensley, K., Benaksas, E.J., Bolli, R., Comp, P., Grammas, P., Hamdheydari, L., Mou, S., Pye, Q.N., Stoddard, M.F., Wallis, G., Williamson, K.S., West, M., Wechter, W.J., Floyd, R.A., 2004. New perspectives on vitamin E: gamma-tocopherol and carboxyelthylhydroxychroman metabolites in biology and medicine. Free Radic. Biol. Med. 36, 1–15.

Jiang, D., Jha, N., Boonplueang, R., Andersen, J.K., 2001. Caspase 3 inhibition attenuates hydrogen peroxide-induced DNA fragmentation but not cell death in neuronal PC12 cells. J. Neurochem. 76, 1745–1755.

Kehrer, J.P., 1993. Free radicals as mediators of tissue injury and disease. Crit. Rev. Toxicol. 23, 21–48.

Kim, H.C., Jhoo, W.K., Kim, W.K., Suh, J.H., Shin, E.J., Kato, K., Ho Ko, K., 2000. An immunocytochemical study of mitochondrial manganese-superoxide dismutase in the rat hippocampus after kainate administration. Neurosci. Lett. 281, 65–68.

Kidd, P.M., 2009. Integrated brain restoration after ischemic stroke – medical management, risk factors, nutrients, and other interventions for managing inflammation and enhancing brain plasticity. Altern. Med. Rev. 14, 14–35.

Klein, J.A., Ackerman, S.L., 2003. Oxidative stress, cell cycle, and neurodegeneration. J. Clin. Invest. 111, 785–793.

Korotkich, I., Senikiene, Z., Simoniene, G., Lazauska, R., Laukeviciene, A., Kevelaitis E., 2006. Inotropic and lusitropic effects of Perilla frutescens (L.) Britton extract on the rabbit myocardium. Medicina 42, 406–412.

Kolosova, N.G., Shcheglova, T.V., Sergeeva, S.V., Loskutova, L.V., 2006. Long-term antioxidant supplementation attenuates oxidative stress markers and cognitive deficits in senescent-accelerated Oxys rats. Neurobiol. Aging 27, 1289–1297.

Kovacic, P., Pozos, R.S., Somanathan, R., Shangari, N., O'Brien, P.J., 2005. Mechanism of mitochondrial uncouplers, inhibitors, and toxins: focus on electron transfer, free radicals, and structure-activity relationships. Curr. Med. Chem. 12, 2601–2623.

Lewen, A., Matz, P., Chan, P.H., 2000. Free radical pathways in CNS injury. J. Neurotrauma 17, 871–890.

Li, H., Repa, J.J., Valasek, M.A., Beltroy, E.P., Turley, S.D., German, D.C., Dietschy, J.M., 2005. Molecular, anatomical, and biochemical events associated with neurodegeneration in mice with Niemann-pick type C disease. J. Neuropathol. Exp. Neurol. 64, 323–333.

López, E., Arce, C., Oset-Gasque, M.J., Cañadas, S., González, M.P., 2006. Cadmium induces reactive oxygen species generation and lipid peroxidation in cortical neurons in culture. Free Radic. Biol. Med. 40, 940–951.

Meng, L., Lozano, Y.F., Gaydou, E.M., Li, B., 2008. Antioxidant activities of polyphenols extracted from Perilla frutescens varieties. Molecules 14, 133–140.

Osakabe, N., Yasuda, A., Natsume, M., Yoshikawa, T., 2004. Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine twostage skin model. Carcinogenesis 25, 549–557.

Ragažinskienė, O., Gailys, V., Jankauskiene, K., Simoniene, G., Jurkstiene, V., 2004. Common Perilla (Perilla frutescens (L.) Britton.) as a perspective immunomodulator. Medicina 40, 220–224.

Riebeling, C., Forsea, A.M., Raisova, M., Orfanos, C.E., Geilen, C.C., 2002. The bisphosphonate pamidronate induces apoptosis in human melanoma cells in vitro. Br. J. Cancer 87, 366–371.

Sanbongi, C., Takano, H., Osakabe, N., Sasa, N., Natsume, M., Yanagisawa, R., Inoue, K.I., Sadakane, K., Ichinose, T., Yoshikawa, T., 2004. Rosmarinic acid in Perilla extract inhibits allergic inflammation induced by mite allergen, in a mouse model. Clin. Exp. Allergy 34, 971–977.

Schmeda-Hirschmann, G., Rodriguez, J.A., Theoduloz, C., Astudillo, S.L., Feresin, G.E., Tapia, A., 2003. Free-radical scavengers and antioxidants from Peumus boldus Mol. (Boldo). Free Radic. Res. 37, 447–452.

Simon, H.U., Haj-Yehia, A., Levi-Schaffer, F., 2000. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5, 415–418.

Singer, C.A., Figueroa-Masot, X.A., Batchelor, R.H., Dorsa, D.M., 1999. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J. Neurosci. 19, 2455–2463.

Sun, Y., Dong, Y., Jiang, H.J., Cai, T.T., Chen, L., Zhou, X., Chen, T., Xu, Q., 2009. Dissection of the role of paeoniflorin in the traditional Chinese medicinal formula Si–Ni-San against contact dermatitis in mice. Life Sci. 84, 337–344.

Swerdlow, R.H., 2007. Treating neurodegeneration by modifying mitochondria: potential solutions to a "complex" problem. Antioxid. Redox Signal. 9, 1591–1603.

Takano, H., Osakabe, N., Sanbongi, C., Yanagisawa, R., Inoue, K., Yasuda, A., Natsume, M., Baba, S., Ichiishi, E., Yoshikawa, T., 2004. Extract of *Perilla frutescens* enriched for rosmarinic acid, a polyphenolic phytochemical, inhibits seasonal allergic rhinoconjunctivitis in humans. Exp. Biol. Med. Maywood 229, 247–254.

Tammariello, S.P., Quinn, M.T., Estus, S., 2000. NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons. J. Neurosci. 20, 1–5.

Theoharides, T.C., 2009. Luteolin as a therapeutic option for multiple sclerosis. J. Neuroinflammation 6, 29.

Tsai, F.S., Peng, W.H., Wang, W.H., Wu C.R., Hsieh, C.C., Lin, Y.T., Feng, I.C., Hsieh, M.T., 2007. Effects of luteolin on learning acquisition in rats: involvement of the central cholinergic system. Life Sci. 80, 1692–1698.

Ueda, H., Yamazaki, C., Yamazaki, M., 2003. Inhibitory effect of Perilla leaf extract and luteolin on mouse skin tumor promotion. Biol. Pharm. Bull. 26, 560–563.

van Meeteren, M.E., Hendriks, J.J., Dijkstra, C.D., van Tol, E.A., 2004. Dietary compounds prevent oxidative damage and nitric oxide production by cells involved in demyelinating disease. Biochem. Pharmacol. 67, 967–975.

Van Zanden, J.J., Geraets, L., Wortelboer, H.M., van Bladeren, P.J., Rietjens, I.M., Cnubben, N.H., 2004. Structural requirements for the flavonoid-mediated modulation of glutathione S-transferase P1-1 and GS-X pump activity in MCF7 breast cancer cells. Biochem. Pharmacol. 67, 1607–1617.

Vural, M., Arslantaş, A., Yazihan, N., Köken, T., Uzuner, K., Arslantaş, D., Ozbek, Z., 2009. NMDA receptor blockage with 2-amino-5-phosphonovaleric acid improves oxidative stress after spinal cord trauma in rats. Spinal Cord, doi:10.1038/sc.2009.100.

Wang, L., Zhou, G.B., Liu, P., Song, J.H., Liang, Y., Yan, X.J., Xu, F., Wang, B.S., Mao, J.H., Shen, Z.X., Chen, S.J., Chen, Z., 2008. Dissection of mechanisms of Chinese medicinal formula Realgar-Indigo naturalis as an effective treatment for promyelocytic leukemia. Proc. Natl. Acad. Sci. U. S. A. 105, 4826–4831.

Wu, J., Liu, T., Xie, J., Xin, F., Guo, L., 2006. Mitochondria and calpains mediate caspase-dependent apoptosis induced by doxycycline in HeLa cells. Cell. Mol. Life Sci. 63, 949–957.

Yamamoto, H., Ogawa, T., 2002. Antimicrobial activity of Perilla seed polyphenols against oral pathogenic bacteria. Biosci. Biotechnol. Biochem. 66, 921–924.

Yoshioka, M., Tanaka, K., Miyazaki, I., Fujita, N., Higashi, Y., Asanuma, M., Ogawa, N., 2002. The dopamine agonist cabergoline provides neuroprotection by activation of the glutathione system and scavenging free radicals. Neurosci. Res. 43, 259–267.

Zhang, H.Y., Yang, D.P., Tang, G.Y., 2006. Multipotent antioxidants: from screening to design. Drug Discov. Today 11, 749–754.

Zhao, G., Qin, G.W., Wang, J., Chu, W.J., Guo, L.H., 2009. Functional activation of monoamine transporters by luteolin and apigenin isolated from the fruit of Perilla frutescens (L.) Britt. Neurochem. 56, 168–176.

Zhao, G., Zang, S.Y., Zheng, X.W., Zhang, X.H., Guo, L.H., 2008. Bakuchiol analogs inhibit monoamine transporters and regulate monoaminergic functions. Biochem. Pharmacol. 75, 1835–1847.