

Quercetin inhibition of ROS-dependent and -independent apoptosis in rat glioma C6 cells

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Abstract

In the present study, we investigated the protective mechanism of quercetin (QUE) and its glycosides, rutin (RUT) and quercitrin (QUI), on reactive oxygen species (ROS)-dependent (H_2O_2) and -independent (chemical anoxia) cell death in rat glioma C6 cells. Induction of HO-1 protein expression was detected in QUE- but not RUT- or QUI-treated C6 cells, and this was prevented by cycloheximide and actinomycin D. Incubation of C6 cells with QUE, but not RUT or QUI, protected C6 cells from H_2O_2 - and chemical anoxia-induced cytotoxicity according to the MTT and LDH release assays. Apoptotic characteristics including chromatin condensation, DNA ladders, and hypodiploid cells appeared in H_2O_2 - and chemical anoxia-treated C6 cells, and those events were significantly suppressed by adding QUE (but not RUT or QUI). Increases in caspase 3, 8, and 9 enzyme activities with decreases in pro-PARP and pro-caspase 3 protein levels and an increase in cleaved D4-GDI protein were identified in H_2O_2 - and chemical anoxia-treated C6 cells, and these were blocked by the addition of QUE, but not by RUT or QUI. Intracellular peroxide levels increased with H_2O_2 and decreased with chemical anoxia, and the addition of QUE reduced the intracellular peroxide levels induced by H_2O_2 . Results of an anti-DPPH radical assay showed that QUE, RUT, and QUI dose-dependently inhibited the production of DPPH radicals in vitro; however, QUE (but not RUT or QUI) prevention of DNA damage induced by OH radicals was identified with a plasmid digestion assay. Increases in phosphorylated ERK and p53 protein expressions were detected in H_2O_2 - but not chemical anoxia-treated C6 cells, and the addition of QUE significantly blocked H_2O_2 -induced phosphorylated ERK and p53 protein expressions. Adding the HO-1 inhibitors, SnPP, CoPP, and ZnPP, reversed the protective effect of QUE against H_2O_2 - and chemical anoxia-induced cell death according to the MTT assay and morphological observations. Additionally, QUE exhibited inhibitory effects on LPS/TPA-induced transformation in accordance with a decrease in MMP-9 enzyme activity and iNOS protein expression in C6 cells. Taken together, the results of this study suggest that QUE exhibits an inhibitory effect on both ROS-dependent and -independent cell death, and induction of HO-1 protein expression is involved.

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Keywords: Quercetin; ROS; Chemical anoxia; HO-1; Apoptosis

Abbreviations: HO-1, Heme oxygenase 1; LPS, Lipopolysaccharide; SnPP, Tin protoporphyrin; iNOS, Inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NBT, Nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; ZnPP, Zinc protoporphyrin; CoPP, Cobalt protoporphyrin; QUE, Quercetin; RUT, Rutin; QUI, Quercitrin; ERKs, Extracellular regulated kinases; MMP-9, Metalloproteinase-9; TPA, 12-O-tetradecanoylphorbol-13-acetate; DCF, 2',7'-dichlorofluorescein; ROS, Reactive oxygen species

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1. Introduction

Several lines of evidence have implicated the role of reactive oxygen species (ROS) in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Adams and Odunze, 1991; Smith et al., 1994; Petersen et al., 1999). ROS may be the initial molecules leading to apoptosis, and hydrogen peroxide (H_2O_2) is one of the major oxygen species which mediate cytotoxicity (Lander, 1997; Ko et al., 2004). H_2O_2 easily penetrates cell membranes and produces deleterious effects within original cells or neighboring cells (Halliwell, 1992). In addition, ROS-independent cell death has also been reported, and cell death was found in ischemic tissues such as those of the brain and kidneys (Chen et al., 2002; Kuan et al., 2003). A reduction in the supplies of glucose and oxygen to the brain leads to neuronal death (Pringle et al., 1997). Horn et al. (2005) suggested that oxygen and glucose deprivation induces apoptosis in the hippocampus, and Kuan et al. (2003) detected brain injury induced by ischemia–hypoxia in mice. Therefore, agents with the ability to protect cells from both H_2O_2 - and ischemia/anoxia-induced cell death may possess the beneficial potential for further development.

Natural food-derived components have received great attention in recent years, and several biological activities including antioxidant, apoptosis-induction, and anti-inflammation have been identified (Shen et al., 2004; Ko et al., 2005; Lin et al., 2005). Flavonoids are a large heterogeneous group of benzo- γ -pyron derivatives, which are present in fruits, vegetables, and Chinese herbs. It has been proposed that flavonoids exert positive health effects in cancer and neurodegenerative disorders, which are attributed to their free radical-scavenging activities (for a review, see Rice-Evans, 2001). Quercetin (QUE), a major dietary flavonoid occurring in apples, onions, and tea, has been extensively investigated. Ranelletti et al. (1992) indicated that QUE inhibits proliferation of colon cancer cells and primary colorectal tumors via blocking the expression of p21-RAS. Ishikawa and Kitamura (2000) suggested that QUE was able to protect mesangial cells from H_2O_2 -induced cell death via the intervention of JNK and ERK activation. Our previous studies showed that QUE induces apoptosis in human leukemia HL-60 cells via activation of the caspase 3 cascade (Shen et al., 2003). Although protection against oxidative stress-induced cell death by QUE has been reported, its effects on both ROS-dependent and -independent cell death in glioma cells are still unclear.

Heat shock proteins (HSPs) are a family of proteins in cellular defense systems which confer cyto-

protection against several extracellular insults such as ischemia/reperfusion and ROS stimulation (Fan et al., 2005; Lee et al., 2005). Experimental upregulation of intracellular HSP levels can protect cells from ischemia and oxidative stress-induced DNA damage and cell death (Beere et al., 2000). This suggests that non-toxic inducers of HSP expression might be used as pharmacological agents for real protection of cells against ischemia and oxidative stress challenges. Heme oxygenase (HO) is a microsomal enzyme used to catalyze the degradation of heme groups to yield biliverdin, iron, and carbon monoxide (CO). HO-1 is an inducible enzyme in response to several inducers such as cytokines, mitogens, nitric oxide (NO), and chemicals. In nervous systems, HO-1 can be induced in glia, and increased HO-1 gene expression has been reported in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (for a review see Schipper, 2004). Several previous studies indicated that HO-1 induction protects tissues against a wide range of injuries induced by hypoxic, cyclosporine A, and inflammation (Liu et al., 2003; Rezzani et al., 2005). Our previous study demonstrated that HO-1 induction participates in protecting macrophages from H_2O_2 -induced apoptosis (Chow et al., 2005). However, the role of HO-1 gene expression in QUE's protection of glial cells from ROS-dependent and -independent cell death is still unclear.

In the present study, we demonstrate that QUE, but not its glycosides (rutin and quercitrin), protects glioma C6 cells from H_2O_2 - and chemical anoxia-induced cell death. Induction of HO-1 protein expression and the differential activation of ERKs, p53, and intracellular peroxide production were investigated.

2. Methods

2.1. Cells

Rat glioma C6 cells were obtained from American Type Culture Collection, incubated in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% heat-inactivated fetal bovine serum, and maintained at 37 °C in a humidified incubator containing 5% CO_2 .

2.2. Agents

The structurally related flavonoids, including QUE, quercitrin (QUI), and rutin (RUT), as well as (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (MTT), hydrogen peroxide (H_2O_2), tin protoporphyrin (SnPP), copper protoporphyrin (CoPP), zinc protoporphyrin (ZnPP), actinomycin D, cycloheximide, 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), and propidium iodine (PI), were

obtained from Sigma Chemical (St. Louis, MO). The antibodies of anti-HO-1, anti- α -tubulin, anti-pERK, anti-HO-2, anti-iNOS, and anti-HSP90 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). PD98059 was obtained from Calbiochem (San Diego, CA).

2.3. Western blotting

Total cellular extracts were prepared according to our previous paper, separated on 8–12% SDS-polyacrylamide minigels, and transferred to immobilon polyvinylidenedifluoride membranes (Millipore). Membranes were incubated with 1% bovine serum albumin and then incubated with specific antibodies overnight at 4 °C. Expression of protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

2.4. Determination of ROS production

The production of ROS was monitored by flow cytometry using DCHF-DA. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped within cells. Hydrogen peroxide or low-molecular-weight hydroperoxides produced by cells oxidize DCHF to the highly fluorescent compound, 2',7'-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by cells. In the present study, cells were treated with QUE, QUI, RUT, or NAC in the presence of H₂O₂ for 2 h. Then compound-treated cells were washed twice with PBS to remove the extracellular compounds, and DCHF-DA (100 μ M) green fluorescence was added, excited using an argon laser, and detected using a 525-nm (FL1-H) band-pass filter by flow cytometric analysis.

2.5. Cell viability assay

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well into 24-well plates for 12 h, followed by treatment with different concentrations of each compound for a further 12 h. Cells were washed with PBS three times, and MTT (50 mg/mL) was added to the medium for 4 h. Then, the supernatant was removed, and the formazone crystals were dissolved using 0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories Inc., Chantilly VA, USA). Data on cellular viability were expressed as a percentage of the control (surviving control cells) in the present study.

2.6. LDH release assay

The percentage of LDH released was expressed as a proportion of the LDH released into the medium compared to the total amount of LDH present in cells treated with 2% trito-

100. The activity was monitored as the oxidation of NADH at 530 nm by an LDH assay kit (Roche, Mannheim, Germany).

2.7. DNA gel electrophoresis

Cells under different treatments were collected, washed with PBS twice, and lysed in 80 μ L of lysis buffer (50 mM Tris (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA); 0.5% sodium sarkosinate, and 1 mg/mL proteinase K) for 3 h at 56 °C and then treated with 0.5 mg/mL RNase A for another hour at 56 °C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point agarose, and 0.025% (w/w) bromophenol blue) and loaded onto pre-solidified 2% agarose gels containing 0.1 mg/mL ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer. Gels were observed and photographed under UV light.

2.8. Flow cytometric analysis

Cells were treated with the indicated compounds for a further 12 h. Trypsinized cells were washed with ice-cold PBS and then incubated [?] in 70% ethanol at –20 °C for at least 1 h. After fixation, cells were washed twice, suspended in 0.5 ml 0.5% Triton X-100/PBS at 37 °C for 30 min with 1 mg/ml of RNase A, and stained with 0.5 ml of 50 mg/ml propidium iodide (PI) for 10 min. Fluorescence emitted from the propidium (PI)-DNA complex was quantified after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickinson, San Jose, CA).

2.9. Caspase activity assay

After different treatments, cells were collected and washed three times with PBS and resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 10 mM ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 15,000 rpm for 3 min, and clear lysates containing 100 μ g of protein were incubated with 100 μ M enzyme-specific colorimetric substrates including Ac-DEVD-pNA for caspase 3/CPP32 at 37 °C for 1 h. Alternative activity of caspase 3 was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

2.10. Plasmid digestion assay

Covalently closed circular plasmid pBR322 DNA (0.25 μ g) in a final volume of 30 μ L was treated with varying concentrations of QUE, RUT, and QUI (12.5, 25, 50, 100 μ M) in the presence of Cu(II) ions and exposed to white light for 2 h. To this, 6 μ L of 5X tracking dye (40 mM EDTA, 0.05% bromophenol blue, and 50% glycerol) was added and loaded onto 0.8% agarose gels. The gel was run at 50 mA and stained with ethidium bromide (0.5 μ g/ml), for 30 min at 4 °C. After washing, the bands were visualized on a UV transilluminator and photographed.

2.11. Anti-DPPH radical assay

The scavenging activities of QUE, RUT, and QUI against the DPPH radical were measured. Briefly, different concentrations of QUE, RUT, and QUI were added to 0.1 ml of 1 M Tris–HCl buffer (pH 7.9) and then mixed with 1.2 ml of 500 μ M DPPH in methanol for 20 min while being protected from light. The absorbance at 517 nm was determined. Deionized water was used as a control group. The decrease in absorbance at 517 nm was calculated to indicate scavenging activity.

2.12. Gelatin zymography

The enzymatic activity of MMP proteins was determined by gelatin zymography. Briefly, after a 24-h incubation of glioma cells with LPS, TPA, or LPS/TPA, aliquots of the conditioned media were mixed with loading buffer, and proteins were separated under non-reduced conditions in 10% polyacrylamide gels containing 1 mg/ml gelatin (Sigma). Then, the gels were washed with 2.5% Triton X-100 and incubated at 37 °C for 24 h in 50 mM Tris–HCl (pH 7.5) buffer containing 0.15 M NaCl, 5 mM CaCl_2 , 1 mM ZnSO_4 , and 40 mmol/L NaN_3 . After staining with Coomassie brilliant blue, MMP activity was evident as clear bands against a blue background.

2.13. Morphological transformation

Glioma C6 cells (10^4 /well) were treated with LPS plus TPA in the presence of different doses of QUE for 24 h, and then cells were fixed with ethanol, followed by staining with a 10% Giemsa solution and three rinses with water. The transformed foci were detected by microscopic observation.

2.14. Statistical analysis

Values are expressed as the mean $\pm$ S.E. Student's *t* test was used to compare the indicated two groups, and the significance of the difference was described. A *p*-value <0.01 was regarded as indicating a significant difference.

3. Results

3.1. QUE (but not QUI or RUT) induced HO-1, but not HO-2 or HSP90, protein expression in rat glioma C6 cells

The chemical structure of QUE and its glycosides, RUT and QUI, are depicted in Fig. 1. RUT and QUI contain a rutinose and rhamnose at C3 of QUE, respectively (Fig. 1). We further analyzed the differential induction of HO-1 protein expression in QUE-, QUI-, and RUT-treated C6 cells. As illustrated in Fig. 2A, QUE at the doses of 25, 50, and 100 μ M induced HO-1, but not HO-2 or HSP90, protein expression in C6 cells (Fig. 2B). Similarly, QUE at the dose of 25 μ M time-dependently

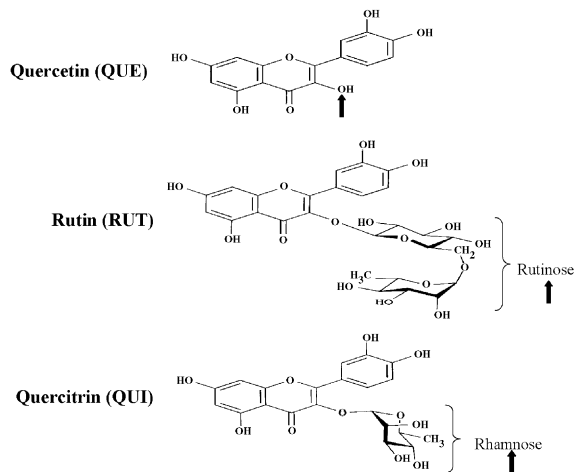


Fig. 1. Chemical structures of quercetin (QUE), quercitrin (QUI), and rutin (RUT).

induced HO-1, but not HO-2 or HSP90, protein expression. Neither QUI nor RUT affected HO-1, HO-2, or HSP90 protein expression (Fig. 2C). Induction of HO-1 protein expression by the HO-1 inducer, hemin, was used as a positive control here. Furthermore, the addition of chemical translational and transcriptional inhibitors, cycloheximide (CHX) and actinomycin D (Act D), significantly attenuated QUE-induced HO-1 protein expression in glioma C6 cells (Fig. 2D). Results of the MTT assay indicated that QUE at a dose of 200 mM exhibited slight but significant cytotoxicity in C6 cells (Fig. 2E). These data suggest that QUE, but not its glycosides, QUI and RUT, is an HO-1 inducer, and de novo protein synthesis is essential for HO-1 protein expression induced by QUE.

3.2. QUE, but not QUI or RUT, protected glioma C6 cells from H_2O_2 - and chemical anoxia-induced cell death via blocking the occurrence of apoptosis

We examined if QUE possesses the ability to protect cells from cell death induced by H_2O_2 and chemical anoxia. In the presence of H_2O_2 and chemical anoxia treatment, a decrease in the viability of C6 cells was detected using the MTT and LDH release assays. Interestingly, pre-incubation of C6 cells with QUE, but not QUI or RUT, significantly inhibited cell death induced by H_2O_2 and chemical anoxia. Results of the MTT assay in Fig. 3A and B show that QUE dose-dependently inhibited the H_2O_2 - and chemical anoxia-induced cytotoxic effects in C6 cells. Similarly, results of the LDH release assay showed that QUE but not its glycosides RUT and QUI at the doses of 25 and 50 μ M attenuated LDH released by H_2O_2 and chemical anoxia treat-

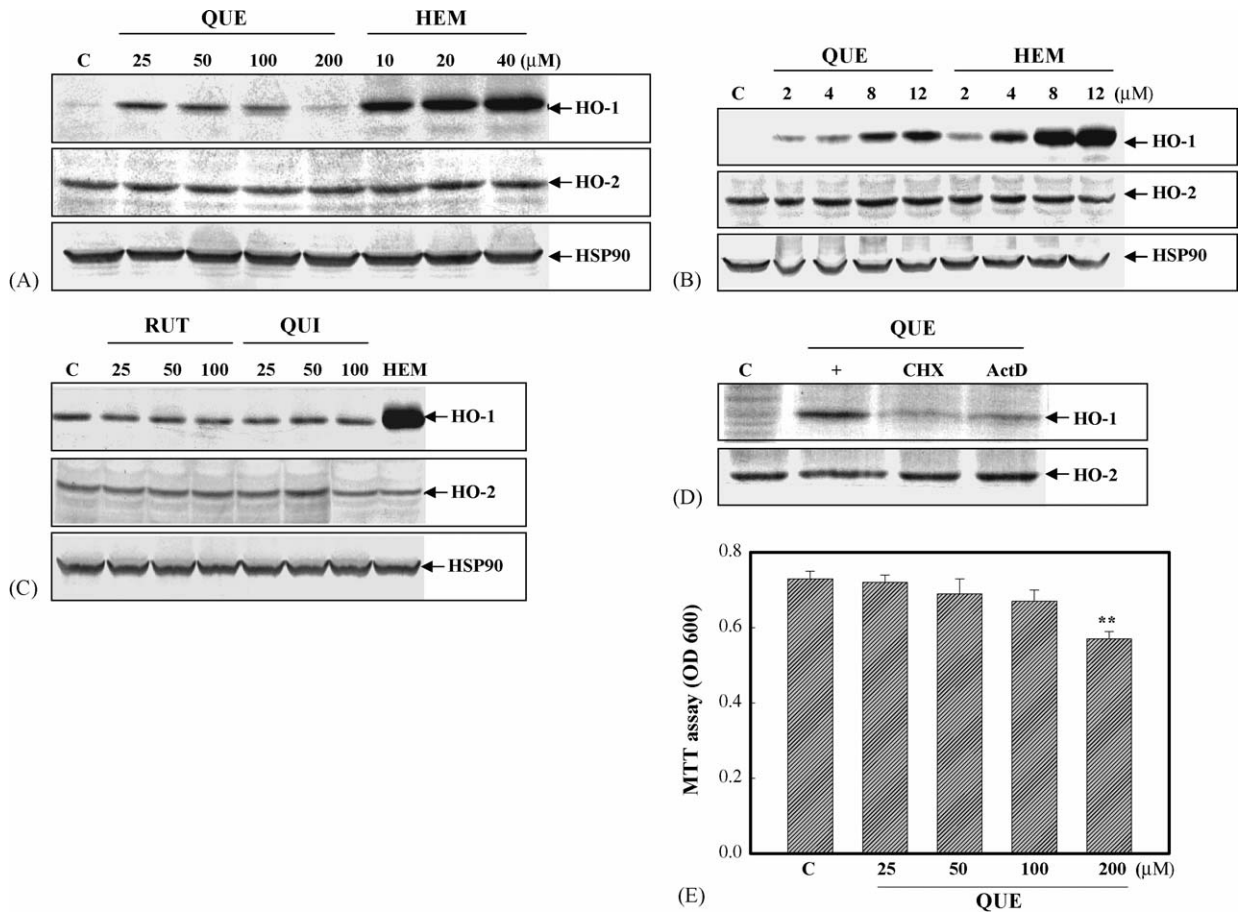


Fig. 2. Quercetin (QUE), but not rutin (RUT) or quercitrin (QUI), induction of HO-1 protein expression in glioma C6 cells. (A) QUE and hemin (HEM) induction of HO-1 protein expression at different doses. Cells were treated with different doses of QUE or HEM for 12 h, and the expressions of HO-1, HO-2, and HSP90 protein were detected by Western blotting. (B) QUE and HEM induction of HO-1 protein expression in a time-dependent manner. Cells were treated with QUE (25 μM) or hemin (10 μM) for different time periods (2, 4, 8, and 12 h), and the expressions of HO-1, HO-2, and HSP90 proteins were analyzed. (C) Neither RUT nor QUI induced HO-1 protein expression in C6 cells. Cell were treated with different doses of RUT or QUI for 12 h, and the expressions of HO-1, HO-2, and HSP90 proteins were analyzed. (D) HO-1 protein induced by QUE was inhibited by actinomycin D (ActD) or cycloheximide (CHX). Cells were treated with QUE (25 μM) in the presence or absence of ActD (1 $\mu\text{g}/\text{mL}$) or CHX (0.25 $\mu\text{g}/\text{mL}$) for 12 h, and the expressions of HO-1 and HO-2 proteins were analyzed. (E) QUE at a dose of 200 μM exhibited cytotoxic effects in C6 cells. C6 cells were treated with different concentrations (25, 50, 100, and 200 μM) of QUE for 12 h. The viability of cells under different treatments was detected by the MTT assay. C, control. Data are expressed as the mean $\pm$ SE. ** $p < 0.01$ indicates a significant difference between indicated groups, as analyzed by Student's t -test.

ment in rat glioma C6 cells (Fig. 3C and D). Furthermore, we examined if QUE's protection against cell death induced by H_2O_2 and chemical anoxia occurred via suppression of apoptosis induction. As illustrated in Fig. 4A, results of morphological observations indicated that apoptotic morphology including the occurrence of condensed chromatin induced by H_2O_2 and chemical anoxia was blocked by QUE but not by QUI or RUT. A loss of DNA integrity via inducing DNA ladders was detected in H_2O_2 - and chemical anoxia-treated cells, which was blocked by the addition of QUE (Fig. 4B). Additionally, the ratios of hypodiploid cells induced by

H_2O_2 and chemical anoxia were significantly reduced by QUE via flow cytometric analysis (Fig. 4C). These data support QUE's protection of C6 cells from H_2O_2 - and chemical anoxia-induced cell death occurring via reducing the incidence of apoptosis.

3.3. QUE suppression of H_2O_2 - and chemical anoxia-induced caspase 3, 8, and 9 enzyme activities in rat glioma C6 cells

Since activation of caspases' enzyme activities has been shown in apoptosis induction, we therefore investi-

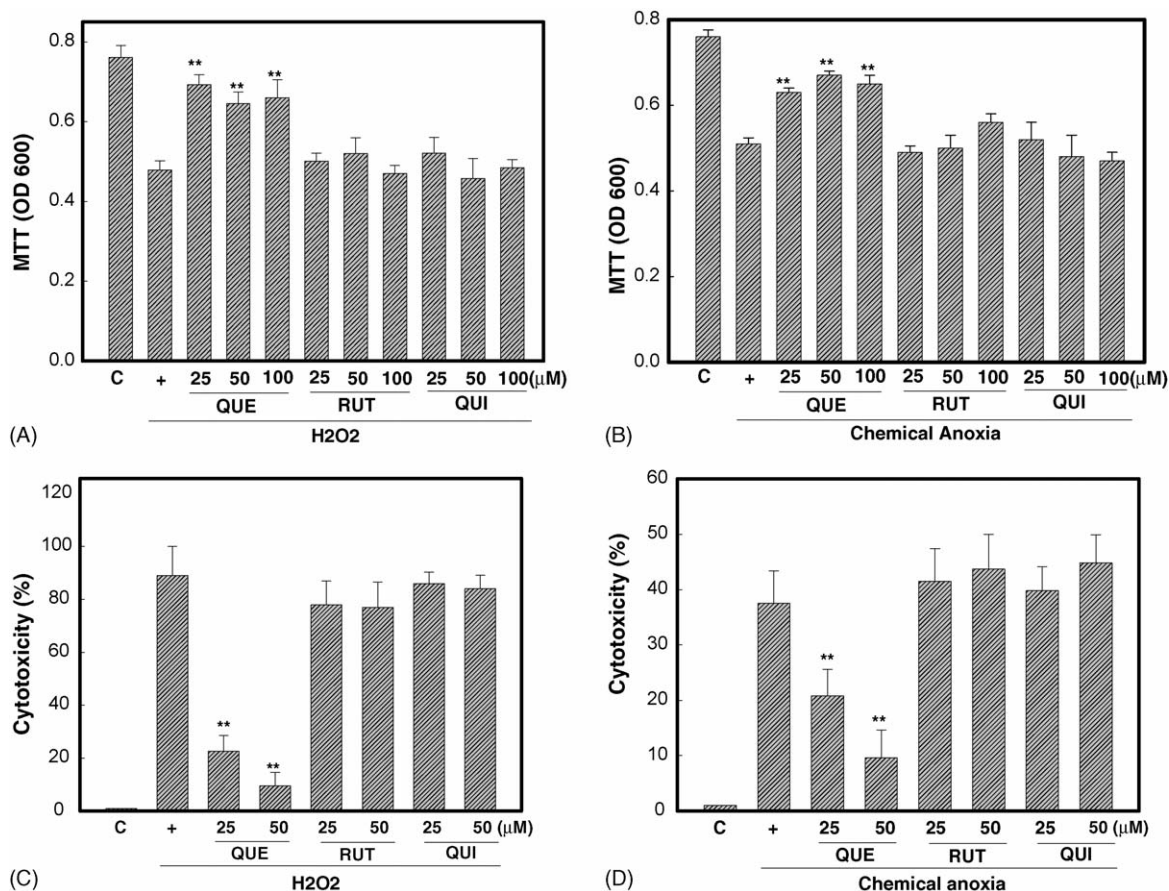


Fig. 3. Quercetin (QUE) protection of glioma C6 cells from H₂O₂- and chemical anoxia-induced cell death by MTT and LDH release assays. (A), (B) QUE (but not quercitrin (QUI) or rutin (RUT)) inhibited H₂O₂- and chemical anoxia-induced cell death according to the MTT assay. Cells were treated with different concentrations (25, 50, and 100 μM) of QUE, QUI, or RUT for 1 h followed by H₂O₂ (400 μM; A) or chemical anoxia (B) treatment for a further 12 h. Cellular viability was detected by the MTT assay as described in "Materials and Methods". (C, D) As described in (A and B), the level of LDH released in the medium was detected using an LDH assay kit as described in "Materials and Methods". Data are expressed as the mean ± SE. ***p* < 0.01 indicates a significant difference between indicated groups, as analyzed by Student's *t*-test.

gated if QUE protection against cell death induced by H₂O₂ and chemical anoxia occurs via blocking caspases' activation. In order to analyze the activities of caspase 3, 8, and 9 enzymes, three specific colorimetric substrates, including a caspase 3 substrate (Ac-DEVD-pNA), a caspase 8 substrate (Ac-IETD-pNA), and a caspase 9 substrate (Ac-LEHD-pNA), were applied in the present study. Results of Fig. 5A–C show that elevation of caspase 3, 8, and 9 enzyme activities was detected in H₂O₂- and chemical anoxia-treated C6 cells, and the addition of QUE significantly reduced the indicated caspase enzyme activity induced by H₂O₂ and chemical anoxia. Results of Fig. 5D show that a decrease in the levels of the proform of caspase 3 and PARP, and an increase in the cleaved form of the caspase 3 substrate, D4-GDI protein, were detected in H₂O₂- and chemical anoxia-treated C6 cells, and those events were blocked

by QUE addition. This suggests that blocking caspases' enzyme activities may be involved in QUE's protection against cell death induced by H₂O₂ and chemical anoxia in C6 cells.

3.4. QUE inhibition of ROS production by DCHF-DA, plasmid digestion, and an anti-DPPH radical assay

It is important to investigate the effect of QUE on ROS production in the presence or absence of H₂O₂ or chemical anoxia treatment. First, the intracellular peroxide level was detected by flow cytometric analysis using DCHF-DA as a fluorescence substrate. As described in Fig. 6A, a significant increase and a slight but significant decrease in intracellular peroxide levels were respectively detected in C6 cells under H₂O₂ and

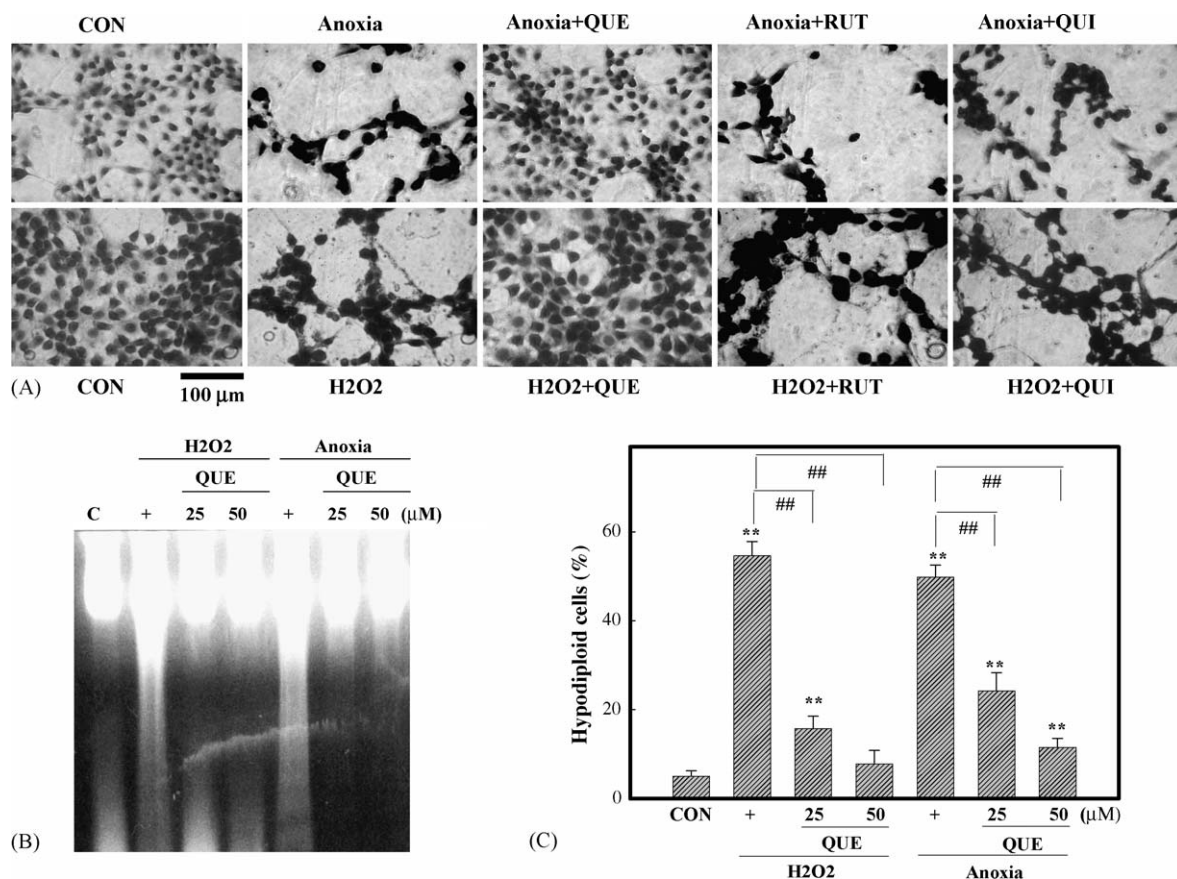


Fig. 4. Quercetin (QUE) inhibition of cell death induced by H₂O₂ or chemical anoxia via blocking the occurrence apoptosis. (A) QUE inhibited apoptotic morphology (condensed cells) induced by H₂O₂ or chemical anoxia as detected by Giemsa staining with microscopic observation. C6 cells were treated as described previously (QUE, rutin (RUT), and quercitrin (QUI) at 25 μM), and the morphology of C6 cells under different treatment was observed microscopically. (B) QUE inhibited DNA ladder formation induced by H₂O₂ or chemical anoxia in C6 cells. Cells were treated with QUE (25 and 50 μM) for 1 h followed by H₂O₂ or chemical anoxia incubation for 12 h. The integrity of DNA was analyzed as described in "Materials and Methods". (C) QUE reduced the ratio of hypodiploid cells in C6 cells according to the flow cytometric analysis. Cells were treated as in (B), and the level of hypodiploid cells under different treatments was analyzed flow cytometric analysis via PI staining.

chemical anoxia treatment. QUE addition reduced intracellular peroxide levels in H₂O₂- and chemical anoxia-treated C6 cells. Neither RUT nor QUI showed any inhibitory effect in C6 cells under different treatments (Fig. 6A). Additionally, the *in vitro* anti-DPPH radical activities of QUE, RUT, and QUI were examined. Data of Fig. 6B show that QUE, RUT, and QUI dose-dependently inhibited DPPH radical production, with IC₅₀ values of 1.8 ± 0.7 , 20.9 ± 1.9 , and 67.5 ± 2.1 μM, respectively. Furthermore, *in vitro* plasmid digestion was used to examine the differential ROS scavenging effects of QUE, RUT, and QUI in the present study via OH radical production induced by the Fenton reaction. Results of Fig. 6C show that OH radicals produced by the Fenton reaction induced DNA damage, described here as a reduction in the level of the supercoiled (SC) form and the appearance of the open-circle (OC) form of the plas-

mid. Interestingly, QUE, but not RUT or QUI, inhibited the conversion of plasmid from the SC to the OC form induced by OH radicals. These data indicate that QUE exhibits the ability to protect cells from ROS-induced DNA damage via its antioxidant activity.

3.5. Increases in phosphorylated ERK and p53 protein expressions in H₂O₂- but not chemical anoxia-treated C6 cells

We further investigated the differential mechanism in H₂O₂- and chemical anoxia-induced cell death. Results of Fig. 7A show that a time-dependent induction of ERK protein phosphorylation was detected in H₂O₂- but not chemical anoxia-treated C6 cells by Western blotting analysis using specific antibodies for phosphorylated ERK proteins. A similar level of total ERK proteins in

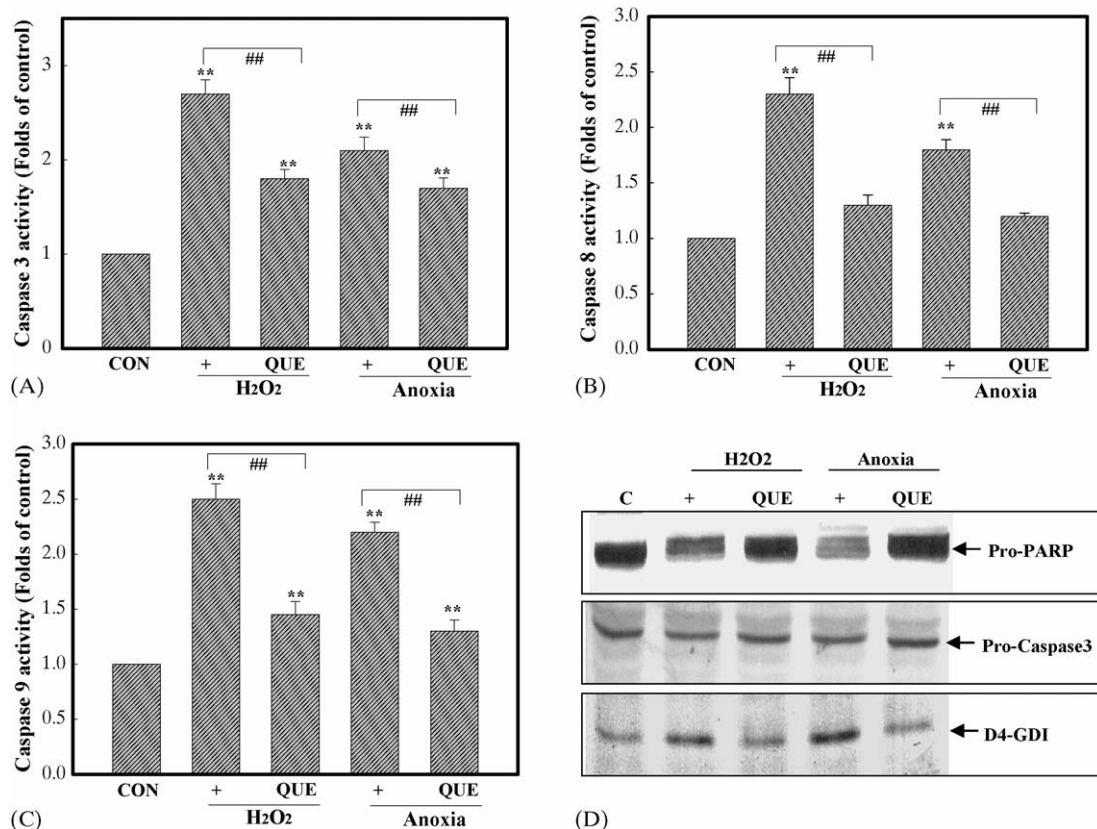


Fig. 5. Quercetin (QUE) inhibited activation of caspase 3, 8, and 9 enzyme activities induced by H₂O₂ and chemical anoxia in glioma C6 cells. The activities of the indicated caspase 3 (A), 8 (B), and 9 (C) were analyzed using specific peptidyl substrates (Ac-DEVD-pNA for caspase 3, Ac-IETD-FMK for caspase 8, and Ac-LEHD-FMK for caspase 9) as described in "Materials and Methods". (D) The expressions of pro-PARP, pro-caspase 3, and cleaved D4-GDI protein under different treatments were detected by Western blotting using specific antibodies. The concentration of QUE was 25 μ M.

each lane was used as an internal control. PD98059, a specific chemical inhibitor of ERKs, was used in the present study to examine if the activation of ERKs is involved in H₂O₂-induced cell death. Results of the MTT assay in Fig. 7B show that PD98059 dose-dependently inhibited H₂O₂- but not chemical anoxia-induced cell death in accordance with reductions in ERK protein phosphorylation. A decrease in the level of phosphorylated ERK proteins induced by H₂O₂ was detected in QUE- but not RUT-treated C6 cells. Both p53-dependent and -independent cell death has been identified, therefore we investigated if the differential induction of p53 protein could be detected in H₂O₂- and chemical anoxia-induced cell death. Results of Fig. 7C indicate that an increase in p53 protein levels was detected in H₂O₂- but not chemical anoxia-treated C6 cells, and this was attenuated by the addition of QUE. The level of α -tubulin protein was used as an internal control here.

3.6. QUE protection of C6 cells from H₂O₂- and chemical anoxia-induced cell death was attenuated by the HO inhibitors, SnPP, CoPP, and ZnPP

In order to identify if the HO-1 protein is involved in the protective mechanism of QUE against H₂O₂- and chemical anoxia-induced cell death, three HO inhibitors, SnPP, CoPP, and ZnPP, were applied in the present study. C6 cells were treated with QUE for 8 h, the indicated HO inhibitor was added for a further 30 min, and H₂O₂ or chemical anoxia treatment followed for a further 12 h. Results of the MTT assay showed that the protective effect of QUE on H₂O₂- or chemical anoxia-induced cell death was blocked by the addition of SnPP, CoPP, and ZnPP (Fig. 8A). Similarly, results of morphological observations indicated that the addition of SnPP, CoPP, and ZnPP induced the re-occurrence of apoptotic morphology in QUE-treated C6 cells subjected to H₂O₂ and chemical anoxia treatment (Fig. 8B).

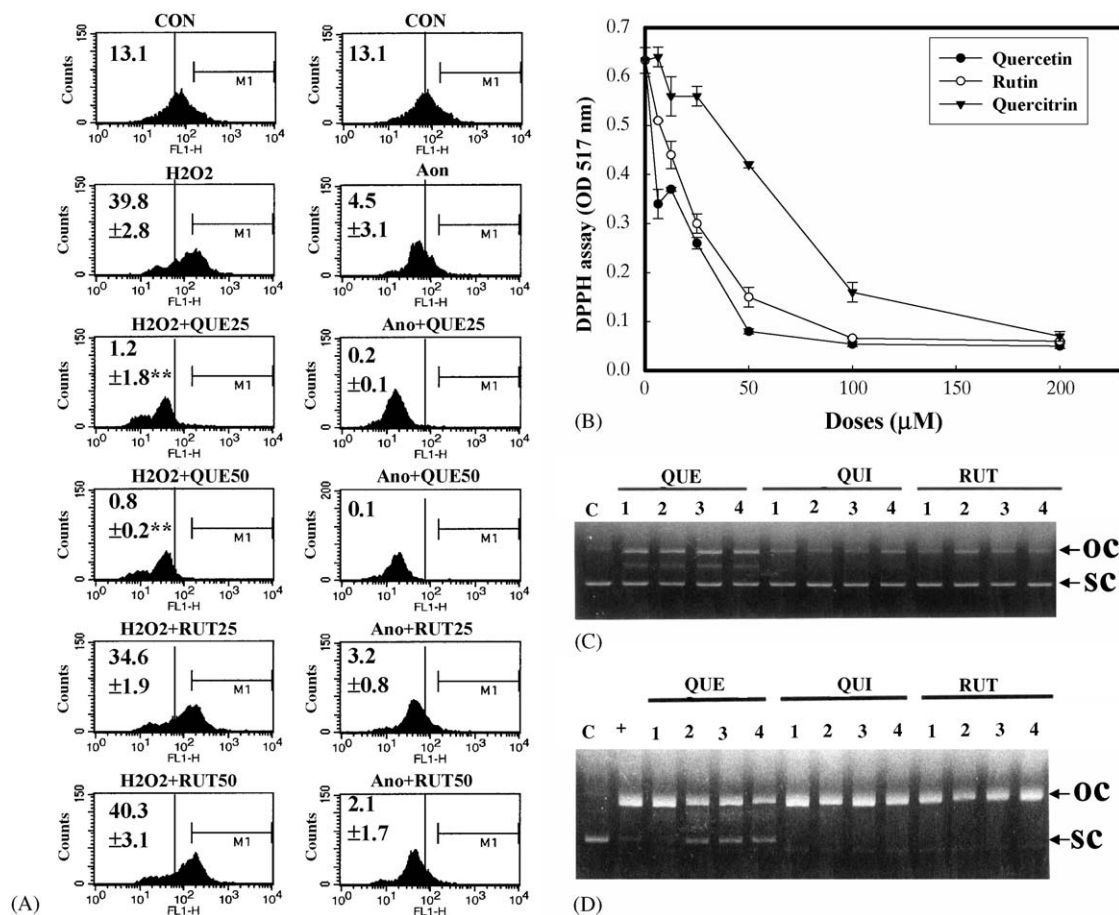


Fig. 6. ROS scavenging activities of quercetin (QUE), rutin (RUT), and quercitrin (QUI) analyzed by DCHF-DA (A), anti-DPPH radical (B), and plasmid digestion (C) assays. (A) Cells were treated with QUE or RUT (25 and 50 mM) for 1 h, followed by incubation with H₂O₂ or chemical anoxia for 4 h. The level of intracellular peroxide was detected by DCHF-DA via flow cytometric analysis. (B) The effects of QUE, RUT, and QUI on DPPH radical production were examined as described in “Materials and Methods”. Additionally, the effects of QUE, RUT, and QUI on OH radical-induced DNA damage was analyzed by a plasmid digestion assay as described in “Materials and Methods”. (C) In the absence of OH radical production, QUE, RUT, and QUI showed no effect on the conformation of the plasmid. (D) In the presence of OH radical production, conversion of plasmid conformation from the SC (supercoiled) to the OC (open circle) form/configuration was detected, and this was blocked by adding QUE, but not RUT or QUI. Data were derived from three independent experiments and are expressed as the mean ± SE. ***p* < 0.01 indicates a significant difference from respective H₂O₂- and chemical anoxia-treated groups, as analyzed by Student’s *t*-test.

3.7. QUE protection of C6 cells from LPS/TPA-induced malignant transformation with reductions in iNOS protein and MMP9 enzyme activities

We earlier established that LPS plus TPA induces malignant transformation in glioma C6 cells by inducing iNOS protein and MMP9 enzyme activities (Chen et al., 2004). Therefore, we investigated if QUE’s protection against ROS-dependent and -independent cell death was in accordance with the blocking of malignant transformation induced by LPS plus TPA. As illustrated in Fig. 9A, LPS and TPA addition induced the occurrence of transformed morphology characterized by the appear-

ance of aggregated cells under microscopic observation via Giemsa staining, which was blocked by the addition of QUE. Results of Western blotting and gelatin zymography indicated that QUE dose-dependently inhibited LPS/TPA-induced iNOS protein expression and MMP-9 enzyme activity (Fig. 9B).

4. Discussion

Because quercetin is a common component of the human diet and possesses several beneficial biological activities, many previous studies have been performed to elucidate its action mechanisms. Data from the present study delineate that quercetin exhibits effective protec-

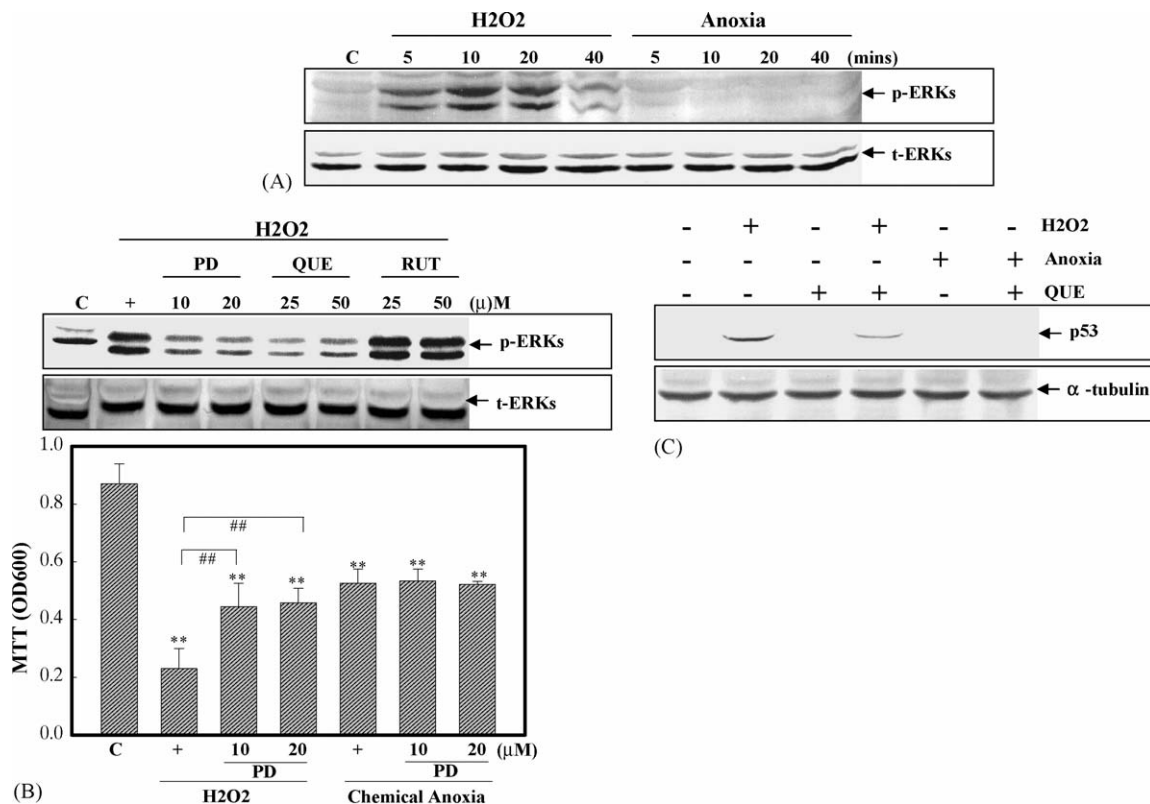


Fig. 7. H₂O₂ but not chemical anoxia induced the phosphorylation of ERK and p53 protein expressions in C6 cells, which were blocked by the addition of quercetin (QUE). (A) C6 cells were treated with H₂O₂ or chemical anoxia for different time periods (5, 10, 20, 40 min), and the expressions of phosphorylated ERK and total ERK proteins were analyzed by Western blotting using specific antibodies. (B) An ERK inhibitor, PD98059 (PD), inhibited the H₂O₂-induced phosphorylation of ERK proteins by blocking H₂O₂- but not chemical anoxia-induced cell death. Cells were treated with PD (10 and 20 μM), QUE (25 and 50 μM), and rutin (RUT; 25 and 50 μM) for 1 h followed by incubation with H₂O₂ for 20 min. The expressions of phosphorylated ERK and total ERK proteins were analyzed by Western blotting using specific antibodies (upper panel). (Lower panel) The viability of C6 cells under different treatments was evaluated with the MTT assay. (C) p53 protein expression induced by H₂O₂ (but not chemical anoxia) was attenuated by adding QUE to glioma C6 cells. As described previously, the expressions of p53 and α-tubulin (as an internal control) proteins were examined by Western blotting.

tive activity against H₂O₂- and chemical anoxia-induced apoptosis in glioma C6 cells. The differential mechanism of QUE's protection in C6 cells characterized by analyzing ROS scavenging activity, and p53 and phosphorylated ERK protein expressions was identified. Additionally, QUE blocked LPS/TPA-induced transformation by reducing iNOS protein expression and MMP-9 enzyme activity in C6 cells. The present data also support glycoside playing a suppressive moiety in QUE's protection against cell death in glioma C6 cells.

HSPs are chaperones that facilitate the correct folding and assembly of nascent polypeptides. In stress conditions such as ischemia or heat shock, HSPs are efficiently translated and reach about 20% of intracellular protein content. The antiapoptotic activities of HSPs have previously been reported. Garrido et al., in a review in 2001 indicated that HSP70 and HSP27 are antiapop-

totic, whereas HSP60 and HSP10 are proapoptotic. The antiapoptotic effect of HSPs is mediated by suppressing caspase activation (Awasthi and Wagner, 2005). QUE has been reported to be an inhibitor of HSP expression, and QUE blocks glial injury by reducing HSP70 protein expression (Wu and Yu, 2000). In HeLa cells, the reduction in HSP expression promotes the induction of apoptosis by QUE, and QUE enhances cisplatin-induced apoptosis via inhibiting HSP70 and multi-drug resistance protein levels (Jakubowicz-Gil et al., 2002, 2005). In a previous study, we demonstrated that QUE is an HO-1 inducer in macrophages (Chow et al., 2005). In the present study, we first identify that HO-1, but not HO-2 or HSP90, protein expression can be induced by QUE, but not QUI or RUT, in glioma C6 cells. QUE protection against H₂O₂- and chemical anoxia-induced cell death was blocked by the HO inhibitors, SnPP, CoPP, and

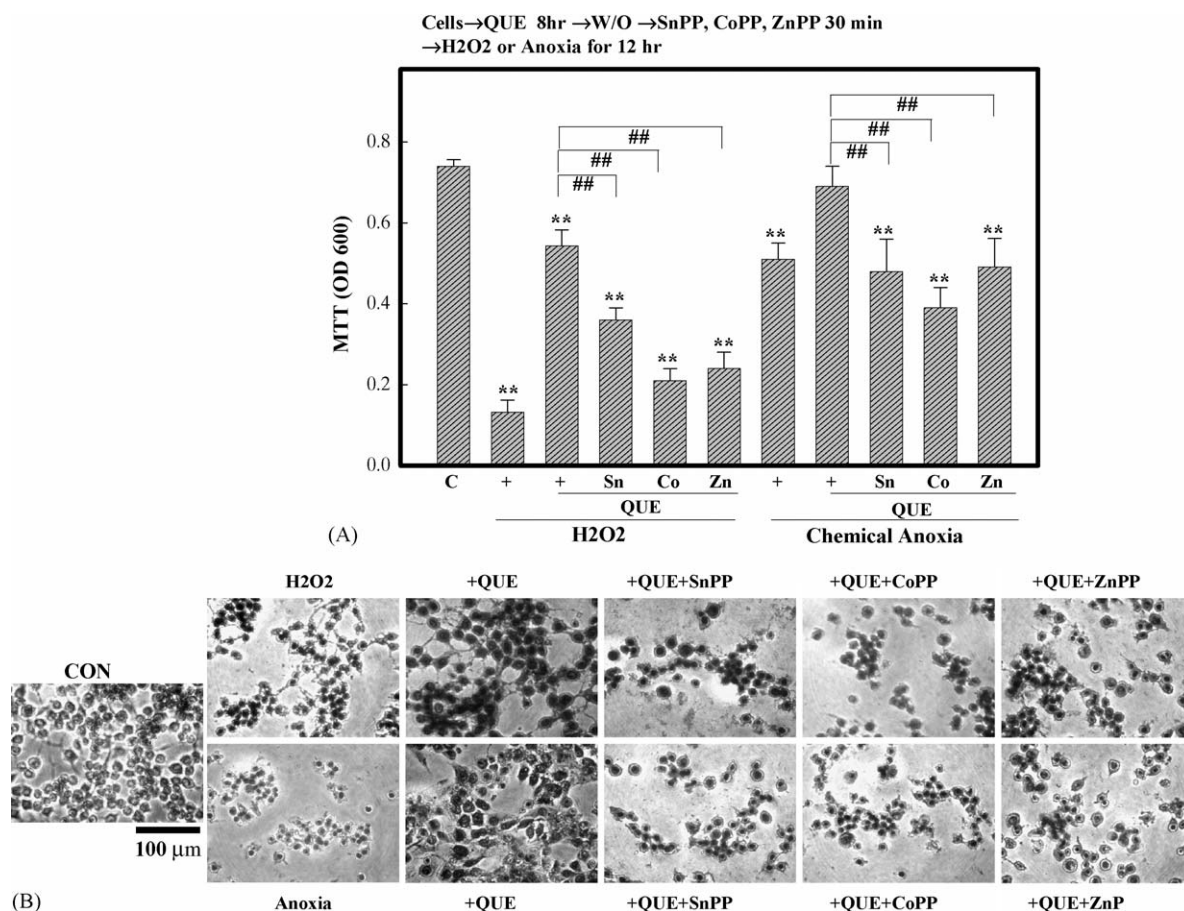


Fig. 8. HO-1 inhibitors SnPP, CoPP, and ZnPP reversed the protective effect of quercetin (QUE) on H₂O₂- and chemical anoxia-induced cell death in glioma C6 cells. (A) C6 cells were treated with QUE (25 μ M) for 8 h, and further incubated with SnPP (1 μ M), CoPP (1 μ M), or ZnPP (0.5 μ M) for a 30 min, followed by H₂O₂ or chemical anoxia treatment for 12 h. The viability of cells under different treatments was detected using the MTT assay. Data were derived from three independent experiments and are expressed as the mean $\pm$ SE. ** $p < 0.01$ indicates a significant difference from the respective H₂O₂- and chemical anoxia-treated groups, as analyzed by Student's *t*-test. (B) As described in (A), the morphological changes under different treatments were examined by microscopic observations of Giemsa staining.

ZnPP. This suggests that HO-1 protein may participate in the protective mechanism of QUE in C6 cells.

The antioxidant and prooxidant activities of QUE have previously been reported. Lapidot et al., indicated that it caused higher production of H₂O₂ and proliferation inhibition in pancreatic β -cell HITs (Lapidot et al., 2002). Jeong et al. (2005) reported that QUE and RUT inhibited lipid peroxidation induced by Cu²⁺-oxidized LDL in cell-free systems and HUVECs. Similarly, QUE reduces lipid peroxidation induced by Fe and Cu via preventing the loss of glutathione (Boadi et al., 2005). In order to elucidate if the antioxidant or prooxidant activity of QUE is involved in QUE's protection of glioma cells from H₂O₂- and chemical anoxia-induced cell death, three ROS-detecting methods including the anti-DPPH radical assay, plasmid digestion assay, and DCHF-DA analysis were applied in the present study. All tested

compounds were effective in reducing DPPH radical production in vitro, and the scavenging potency was in the order of QUE > RUT > QUI. Results of the DCHF-DA analysis showed that an increase and a slight but significant decrease in intracellular peroxide level were respectively detected in H₂O₂- and chemical anoxia-treated glioma C6 cells. QUE caused significant suppression of intracellular peroxide production induced by H₂O₂. Additionally, QUE, but not RUT or QUI, inhibited plasmid damage induced by the production of the OH radical, represented here as a re-occurrence of the supercoiled (SC) form of the plasmid in the presence of QUE (but not RUT or QUI) treatment. Taken together, we propose that QUE's prevention of H₂O₂-induced cell death occurs through its antioxidant activity, which is distinct from its protective action against chemical anoxia-induced cell death.

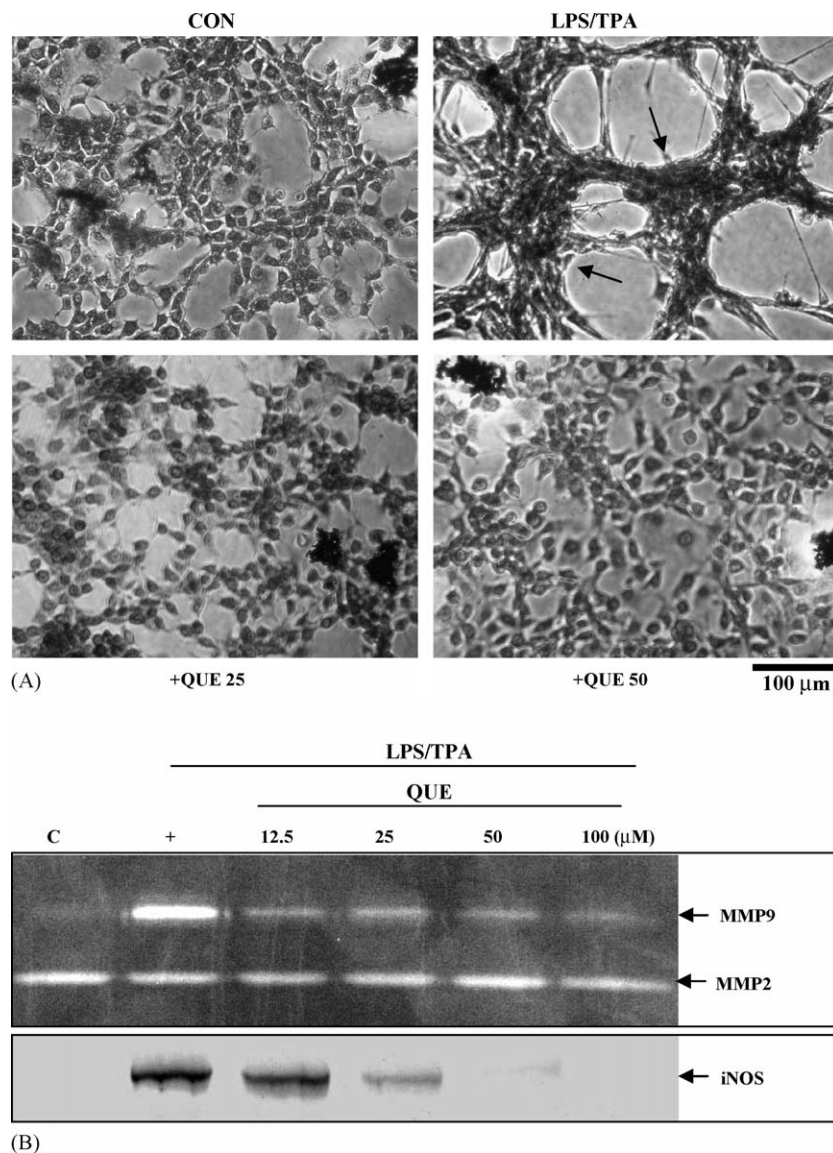


Fig. 9. Quercetin (QUE) prevents C6 cells from LPS/TPA-induced morphological transformation, MMP-9 enzyme activity, and iNOS protein expression. (A) C6 cells were treated with LPS (1 mg/ml) plus TPA (400 ng/ml) for 24 h in the presence or absence of QUE (25 and 50 μM) pretreatment for 1 h. The morphological changes (arrows) were detected microscopically via Giemsa staining. (B) As described in (A), the MMP-2 and MMP-9 enzyme activities released in the medium, and iNOS protein expression in cell lysates were respectively examined by gelatin zymography and Western blotting. An arrow indicates aggregated cells.

Induction of p53 protein expression and ERK protein phosphorylation has been identified in oxidative stress-induced apoptosis. Nair et al. (2004) found that H_2O_2 rapidly induced the phosphorylation of ERK, but not of JNK or p38 kinases, in PC-12-D2R cells. Bonini et al. (2004) suggested that oxidative stress induces p53-mediated apoptosis in glial cells. In A549 cells, H_2O_2 induces Fas upregulation via activation of the PARP-p53 pathway. Data of the present study show that an increase in p53 and phosphorylated ERK protein was detected

in H_2O_2 -treated C6 cells, which was attenuated by the addition of QUE. The addition of a specific chemical inhibitor of ERKs (PD98059) reduced H_2O_2 -induced the phosphorylation of ERK proteins by preventing its cytotoxicity in glioma C6 cells. This suggests that cell death induced by H_2O_2 is mediated by inducing p53 and phosphorylated ERK protein expression in glioma C6 cells. Sodium azide (NaN_3) with the glycolysis blocker, 2-deoxyglucose, has already been used to induce chemical anoxia in cell cultures (Jorgensen et al., 1999). How-

ever, the apoptotic mechanism induced by NaN_3 plus 2-deoxyglucose in glioma cells is still obscure. Results of the present study show that a decrease in intracellular peroxide levels was detected in chemical anoxia-treated C6 cells. However, neither p53 protein nor phosphorylated ERK proteins were induced in chemical anoxia-treated C6 cells. This suggests that distinct apoptotic mechanisms exist in H_2O_2 - and chemical anoxia-treated glioma C6 cells.

Flavonoids extensively exist in foods and it is estimated that the total intake is about 20–1000 mg daily. QUE and its derivatives are the major components of flavonoids in food such as onions, apples, red wine and tea. QUE has a long plasma half-life of 11 to 28 h, and QUE could be accumulated in plasma with repeated intake. Previous study indicated that a 50-mg of QUE would lead to concentrations of up to 0.75 to 1.5 $\mu\text{mole/L}$ in plasma (Scalbert and Williamson, 2000; Manach et al., 2005). Therefore, the doses (25 and 50 μM) of QUE applied in the present study are possibly achieved by repeated daily food consumption. In addition, glycosylated conjugates of flavonoids are predominantly present in plants, fruits and vegetables. Our previous studies have shown that the aglycones have greater biological activities including apoptosis induction and NO inhibition than their respective glycosides (Lin et al., 2005; Chow et al., 2005). Deglycosylation of flavonoid glycosides in mammalian and microbial has been shown before absorption in vivo. In the small intestine and liver, QUE glycosides RUT and QUI as prodrugs can be absorbed and hydrolyzed the sugar at the C3 by cytosolic β -glucosidase such as glucocerebrosidase and lactase phlorizin hydrolase in vivo (Sperket et al., 1997; Cornish et al., 2002). These data suggests a conversion of flavonoid glycosides to aglycones in vivo.

Our previous studies demonstrated that glycoside addition attenuates apoptosis induction and anti-inflammatory activities of flavonoids (Shen et al., 2003; Lin et al., 2005). In the present study, QUE, but not its glycosides RUT or QUI, protected C6 cells from H_2O_2 - and chemical anoxia-induced cell death in accordance with inducing HO-1 protein expression. These data support the option that glycoside plays a negative role in the action of flavonoids in vitro. Malignant transformation is a major cause of mortality in brain tumor patients. Our previous study established a malignant transformation model using glioma C6 cells induced by LPS plus TPA, and activations of MMP-9 enzyme activity and iNOS protein expression are involved. Data of the present study demonstrate that QUE significantly suppresses LPS/TPA-induced morphological transformation, accompanied by reducing MMP-9 enzyme activity

and iNOS protein expression in C6 cells. This suggests that QUE possesses the ability to protect cells from cell death induced by oxidative stress and chemical anoxia and can prevent the malignant transformation induced by LPS plus TPA; it thus has great potential for further development.

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References

- Adams Jr., J.D., Odunze, I.N., 1991. Oxygen free radicals and Parkinson's disease. *Free Radic. Biol. Med.* 10, 161–169.
- Awasthi, N., Wagner, B.J., 2005. Upregulation of heat shock protein expression by proteasome inhibition: an antiapoptotic mechanism in the lens. *Invest. Ophthalmol. Vis. Sci.* 46, 2082–2091.
- Beere, H.M., Wolf, B.B., Cain, K., Mosser, D.D., Mahboubi, A., Kuwana, T., Tailor, P., Morimoto, R.I., Cohen, G.M., Green, D.R., 2000. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat. Cell Biol.* 2, 469–475.
- Boadi, W.Y., Iyere, P.A., Adunyah, S.E., 2005. In vitro exposure to quercetin and genistein alters lipid peroxides and prevents the loss of glutathione in human progenitor mononuclear (U937) cells. *J. Appl. Toxicol.* 25, 82–88.
- Bonini, P., Cicconi, S., Cardinale, A., Vitale, C., Serafino, A.L., Ciotti, M.T., Marlier, L.N., 2004. Oxidative stress induces p53-mediated apoptosis in glia: p53 transcription-independent way to die. *J. Neurosci. Res.* 75, 83–95.
- Chen, T.J., Shen, S.C., Lin, H.Y., Chien, L.L., Chen, Y.C., 2004. Lipopolysaccharide enhancement of 12-O-tetradecanoylphorbol 13-acetate-mediated transformation in rat glioma C6, accompanied by induction of inducible nitric oxide synthase. *Toxicol. Lett.* 147, 1–13.
- Chen, Y.C., Shen, S.C., Lee, W.R., Hsu, F.L., Lin, H.Y., Ko, C.H., Tseng, S.W., 2002. Emodin induces apoptosis in human promyeloleukemic HL-60 cells accompanied by activation of caspase 3 cascade but independent of reactive oxygen species production. *Biochem. Pharmacol.* 64, 1713–1724.
- Chow, J.M., Shen, S.C., Huan, S.K., Lin, H.Y., Chen, Y.C., 2005. Quercetin, but not rutin and quercitrin, prevention of H_2O_2 -induced apoptosis via anti-oxidant activity and heme oxygenase 1 gene expression in macrophages. *Biochem. Pharmacol.* 69, 1839–1851.
- Cornish, K.M., Willisamson, G., Sanderson, J., 2002. Quercetin metabolism in the lens: role in inhibition of hydrogen peroxide induced cataract. *Free Radic. Biol. Med.* 33, 63–70.
- Fan, G.C., Ren, X., Qian, J., Yuan, Q., Nicolaou, P., Wang, Y., Jones, W.K., Chu, G., Kranias, E.G., 2005. Novel cardioprotective role of a small heat-shock protein, Hsp20, against ischemia/reperfusion injury. *Circulation* 111, 1792–1799.

- Garrido, C., Gurbuxani, S., Ravagnan, L., Kroemer, G., 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Commun.* 286, 433–442.
- Halliwell, B., 1992. Reactive oxygen species and the central nervous system. *J. Neurochem.* 59, 1609–1623.
- Horn, A.P., Gerhardt, D., Geyer, A.B., Valentim, L., Cimarosti, H., Tavares, A., Horn, F., Lenz, G., Salbego, C., 2005. Cellular death in hippocampus in response to PI3K pathway inhibition and oxygen and glucose deprivation. *Neurochem. Res.* 30, 355–361.
- Ishikawa, Y., Kitamura, M., 2000. Anti-apoptotic effect of quercetin: intervention in the JNK- and ERK-mediated apoptotic pathways. *Kidney Int.* 58, 1078–1087.
- Jakubowicz-Gil, J., Rzymowska, J., Gawron, A., 2002. Quercetin, apoptosis, heat shock. *Biochem. Pharmacol.* 64, 1591–1595.
- Jakubowicz-Gil, J., Paduch, R., Piersiak, T., Glowniak, K., Gawron, A., Kandefer-Szerszen, M., 2005. The effect of quercetin on pro-apoptotic activity of cisplatin in HeLa cells. *Biochem. Pharmacol.* 69, 1343–1350.
- Jeong, Y.J., Choi, Y.J., Kwon, H.M., Kang, S.W., Park, H.S., Lee, M., Kang, Y.H., 2005. Differential inhibition of oxidized LDL-induced apoptosis in human endothelial cells treated with different flavonoids. *Br. J. Nutr.* 93, 581–591.
- Jorgensen, N.K., Petersen, S.F., Damgaard, I., Schousboe, A., Hoffmann, E.K., 1999. Increases in $[Ca^{2+}]_i$ and changes in intracellular pH during chemical anoxia in mouse neocortical neurons in primary culture. *J. Neurosci. Res.* 56, 358–370.
- Ko, C.H., Shen, S.C., Chen, Y.C., 2004. Hydroxylation at C4' or C6 is essential for apoptosis-inducing activity of flavanone through activation of the caspase 3 cascade and production of reactive oxygen species. *Free Radic. Biol. Med.* 36, 897–910.
- Ko, C.H., Shen, S.C., Lee, T.J.F., Chen, Y.C., 2005. Myricetin inhibits matrix metalloproteinase 2 protein expression and enzyme activity in colorectal carcinoma cells. *Mol. Cancer Ther.* 4, 281–290.
- Kuan, C.Y., Whitmarsh, A.J., Yang, D.D., Liao, G., Schloemer, A.J., Dong, C., Bao, J., Banasiak, K.J., Haddad, G.G., Flavell, R.A., Davis, R.J., Rakic, P., 2003. A critical role of neural-specific JNK3 for ischemic apoptosis. *Proc. Natl. Acad. Sci. USA.* 100, 15184–15189.
- Lander, H.M., 1997. An essential role for free radicals and derived species in signal transduction. *FASEB J.* 11, 118–124.
- Lapidot, T., Walker, M.D., Kanner, J., 2002. Antioxidant and prooxidant effects of phenolics on pancreatic beta-cells in vitro. *J. Agric. Food Chem.* 50, 7220–7225.
- Lee, Y.J., Lee, D.H., Cho, C.K., Chung, H.Y., Bae, S., Jhon, G.J., Soh, J.W., Jeoung, D.I., Lee, S.J., Lee, Y.S., 2005. HSP25 inhibits radiation-induced apoptosis through reduction of PKC δ -mediated ROS production. *Oncogene* 24, 3715–3725.
- Liu, H., Nowak, R., Chao, W., Bloch, K.D., 2003. Nerve growth factor induces anti-apoptotic heme oxygenase-1 in rat pheochromocytoma PC12 cells. *J. Neurochem.* 86, 1553–1563.
- Lin, H.Y., Shen, S.C., Chen, Y.C., 2005. Anti-inflammatory effect of heme oxygenase 1: glycosylation and nitric oxide inhibition in macrophages. *J. Cell. Physiol.* 202, 579–590.
- Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., 2005. Bioavailability and bioefficacy of polyphenols in humans I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 81, 230S–242S.
- Nair, V.D., Yuen, T., Olanow, C.W., Sealfon, S.C., 2004. Early single cell bifurcation of pro- and antiapoptotic states during oxidative stress. *J. Biol. Chem.* 279, 27494–27501.
- Petersen, A., Mani, K., Brundin, P., 1999. Recent advances on the pathogenesis of Huntington's disease. *Exp. Neurol.* 157, 1–18.
- Pringle, A.K., Iannotti, F., Wilde, G.J., Chad, J.E., Seeley, P.J., Sundstrom, L.E., 1997. Neuroprotection by both NMDA and non-NMDA receptor antagonists in in vitro ischemia. *Brain Res.* 755, 36–46.
- Ranelletti, F.O., Ricci, R., Larocca, L.M., Maggiano, N., Capelli, A., Scambia, G., Benedetti-Panici, P., Mancuso, S., Rumi, C., Piantelli, M., 1992. Growth-inhibitory effect of quercetin and presence of type-II estrogen-binding sites in human colon-cancer cell lines and primary colorectal tumors. *Int. J. Cancer* 50, 486–492.
- Rezzani, R., Rodella, L., Bianchi, R., Goodman, A.I., Lianos, E.A., 2005. Protective effects of heme-oxygenase expression in cyclosporine A-induced injury. *Curr. Neurovasc. Res.* 2, 157–161.
- Rice-Evans, C., 2001. Flavonoid antioxidants. *Curr. Med. Chem.* 8, 797–807.
- Scalbert, A., Williamson, G., 2000. Dietary intake and bioavailability of polyphenols. *J. Nutr.* 130, 2073S–2085S.
- Schipper, H.M., 2004. Heme oxygenase expression in human central nervous system disorders. *Free Radic. Biol. Med.* 37, 1995–2011.
- Shen, S.C., Chen, Y.C., Hsu, F.L., Lee, W.R., 2003. Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. *J. Cell. Biochem.* 89, 1044–1055.
- Shen, S.C., Ko, C.H., Tseng, S.W., Chen, Y.C., 2004. Structurally-related anti-tumor effects of flavanones in vitro and in vivo: involvement of caspase 3 activation, p21 gene expression and reactive oxygen species production. *Toxicol. Appl. Pharmacol.* 197, 84–95.
- Smith, M.A., Richey, P.L., Taneda, S., Kutty, R.K., Sayre, L.M., Monnier, V.M., Perry, G., 1994. Advanced Maillard reaction end products, free radicals, and protein oxidation in Alzheimer's disease. *Ann. NY Acad. Sci.* 738, 447–454.
- Sperket, B., Backman, J.T., Kroemer, H.K., 1997. The role of β -glucuronidase in drug disposition and drug targeting in humans. *Clin. Pharmacokinet.* 33, 18–31.
- Wu, B.Y., Yu, A.C., 2000. Quercetin inhibits c-fos, heat shock protein, and glial fibrillary acidic protein expression in injured astrocytes. *J. Neurosci. Res.* 62, 730–736.