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Flavonoids from the leaves of *Diospyros kaki* reduce hydrogen peroxide-induced injury of NG108-15 cells

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Abstract

Flavonoids from the leaves of *Diospyros kaki L* (FLDK-P70) are main therapeutic components of NaoXingQing, which is a novel and patented Traditional Chinese Medicine drug used for the treatment of syndrome of apoplexy for years in China. The present study investigated the effects of FLDK-P70 on hydrogen peroxide (H_2O_2)-induced apoptosis-like damage of NG108-15 cells. Pretreatment of cells with FLDK-P70 alleviated H_2O_2-induced cell injury and apoptosis by upregulating *Bcl-2* expression and improving redox imbalance as indicated by the alleviation of the decline in the intracellular endogenous antioxidants: glutathione and glutathione peroxidase as well as catalase, with the decrease of the leak of lactate dehydrogenase and the reduction of the accumulation of malondialdehyde. These results indicate that FLDK-P70 may be potentially used in the prevention and treatment of ischemia/reperfusion injury and other neurodegenerative disease. Upregulating bcl-2 and improving cellular redox state by FLDK-P70 may play critical roles in attenuating oxidative injury.
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Keywords: Flavonoids; *Diospyros kaki L*; NG108-15 cells; Antioxidant; Apoptosis; *Bcl-2*; H_2O_2

Introduction

Oxidative stress is generally known to be involved in the acute and chronic injury of central nervous system (CNS) and is a major factor in determining the development of neuronal damages

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(Coyle and Puttfarcken, 1993; Facchinetti et al., 1998; Kehrer and Smith, 1994). In acute ischemic strokes and chronic neurodegenerative disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD), damaged neurons commit cellular apoptosis by oxidative stress, which is mainly induced by reactive oxygen species (ROS), involving in superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) (Barinaga, 1998; Facchinetti et al., 1998; Olanow, 1993). These molecules are highly reactive molecules generated predominantly during cellular respiration and normal metabolism by different oxidases, oxygenases, or by auto-oxidation of catecholamines. Defense mechanisms of cells against oxidative damage involve in enzymatic conversion of ROS into less reactive molecules, chelation of transition metal catalysts and detoxification of ROS by antioxidants. Continuous and excessive production of ROS will inevitably lead to expose cells to ROS directly, which subsequendtly react with and damage cellular components such as proteins, lipids, and DNA, as well as form other toxic products from these reactions. ROS finally can lead to later cell death by necrosis and apoptosis (Yassi et al., 2002). H_2O_2, a major ingredient of ROS, has been widely used to induce oxidative stress in vitro (Satoh et al., 1996; Tanaka et al., 2001).

It was reported that there might be a burst of the generation of ROS during cerebral ischemia, especially at the onset of reperfusion after cerebral ischemia (Barinaga, 1998; Yassi et al., 2002). Brain is especially susceptible to the damage caused by oxidative stress because neurons contain enriched polyunsaturated fatty acids and low levels of endogenous antioxidant enzymes (Olanow, 1993). Thus, some of the treatmeants with antioxidants aimed at scavenging free radicals or preventing their generation might be a rational selection for stroke and other neurodegenerative diseases (Yassi et al., 2002).

NaoXingQing (NXQ), a novel and patented Traditional Chinese medicine (TCM) drug made from the extract of the leaves of *Diospyros kaki L,* Dispryosl and Ebenceae, has been used for the treatment of stroke or syndrome of apoplexy in clinic to improve the outcome of ischemia stroke for years in China. Moreover the remedy has been reported to possess significant efficacy and few side-effects in the treatment of stroke patients such as cerebral atherosclerosis, transitory ischemia symdrone, cerebral thrombogenesis, cerebral thrombosis sequela, apoplexy sequela, cerebral embolism etc (Cai and Yang, 2001; Yu et al., 1988). We have recently demonstrated that NXQ significantly protects NG108-15 cells against H_2O_2 induced damage by improving redox disequilibrium and inhibiting apoptosis (Bei et al., 2004).

Flavonoids have recently attracted public attention because flavonoids in tea, fruits and vegetables lessen the risk of cardiovascular diseases (Hertog et al., 1993; Maxwell et al., 1994). Previous studies have demonstrated that the flavonoid glucosides extracted from the leaves of *Diospyros kaki L* could lower blood pressure, increase coronary blood flow in anaesthesized dog and isolated rabbit heart and dilate the rabbit ear vessels (The Pharmaceutical Factory of PLA 58th Hospital, 1973). It was reported that flavonoids isolated from the leaves of *Diospyros kaki* had a hypotensive action in rats and the inhibitory effects on anginotensin-converting enzyme activity (Kameda et al., 1987).

Flavonoids from leaves of *Diospyros kaki L* (FLDK-P70) are main constituents of NXQ, and compose more than 25% of the total components in NXQ extracts. Although there were some reports of the pharmacological effect of NXQ and other flavonoids as mentioned above, little was known about how FLDK-P70 worked in neuronal cells. Here we carried out the present study to examine the effects of FLDK-P70 on H_2O_2-induced NG108-15 cell injury.

Materials and methods

FLDK-P70

FLDK-P70 was the extract of the leaves of *Diospyros kaki L*. supplied by Guangzhou BaiYunShan TCM Pharmaceutical Factory. FLDK-P70 was prepared according to the follwing operations. Leaves of *D. kaki were* boiled with water, concentrated, sedated with 70% alcohol. The collected alcohol solution was evaporated, extracted with acetic acetate, then evaporated. The extract was made into water solution, then added to polyacrylamide column for chromatographic purification by washing with 70% alcohol, the collected efflux was finally concentrated and dried. FLDK-P70 contains more than 77.4% flavonoids including 34.6% quercetin and its glycosides (hyperin, and isoquercitrin etc) as well as 42.7% kaempferol and its glycosides (astragalin etc.), characterized by high performance liquid chromatography (HPLC) and Liquid Chromatography-Mass Spectrum (LC-MS) assays (data not showed).

Rutin was purchased from Sigma.

FLDK-P70 and rutin were dissolved in an aqueous solution of 5% DMSO (Sigma) with double distilled water stored in −20 °C and diluted with serum-free RPMI 1640 medium (GIBCO) before use.

Cell culture and exposure to H_2O_2

NG108-15 cell line, the Neuroblastoma × Glioma hybrid cells were obtained from the Shanghai Cell Institute, Chinese Academy of Science. Cell culture was performed by the method described (Hamprecht et al., 1985). Cells were plated into multiwell plates or dishes (Falcon Co, USA) at a density of 5~8 × 10^5 cells per ml in RPMI 1640 Medium (Gibco, USA) containing 10% fetal bovine serum (Hyclone Co., USA), 100U/ml of Ampicillin, 100 mg/ml of streptomycin, 2 mM L-glutamine and cultured at 37°C in a damped gas mixture of 5% CO_2 /95% air. Cells were tested in the log-phase about 48 hr after plating.

To induce an oxidative stress, H_2O_2 freshly prepared from 30% stock solution (Sigma) before each experiment was added to the culture. The cells were preincubated with FLDK-P70 or rutin 2 hr prior to the addition of H_2O_2. Assays for cell viability, apoptosis, lipid peroxidation and antioxidants were performed 10 hr after the addition of H_2O_2.

MTT assay

The cytotoxicity of FDKL-P70 and H_2O_2 was measured by MTT (3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyltetrazolium bromide, Sigma) reduction method (Hansen et al., 1989). Cells at a density of 5~8 × 10^5/ml were plated into 96-well tissue culture plates (Falcon Co, USA). After about 24 hr, supernatants were substituted by drug-containing medium. After being incubated with FLDK-P70 or rutin and exposure to H_2O_2 for 10 hr, 20 μl of 5 mg/ml MTT was added to each well and the plates were incubated at 37 °C for 4 hr. The supernatant in each well was taken out carefully. Formazan crystals were redissolved in 100 μl DMSO for 10 min with shaking. Each plate was measured instantly on a microplate reader (ELX 800, BIO-TEK Instruments Inc. USA) at a wavelength of 490 nm. The IC_{50} values were computed using SPSS software.

To test the treated effect of flavonoids on H_2O_2-induced injury in NG108-15 cells, we also post-treat the cells with FLDK-P70 or rutin after the addition of H_2O_2 to the cells for 2 hr and assayed the cell viability.

LDH release into culture medium

The supernatants of the cell culture medium were assayed for the lactate dehydrogenase (LDH) release with LDH Kit (Nanjin JianChen Biochem Co), which examined the cell injury.

Detection of apoptotic cells

After being incubated with FLDK-P70 and H_2O_2, cells were collected by centrifugation and washed with PBS and fixed in 4% (V/V) paraformaldehyde at 4°C for 20min. After stained with 10 μg/ml of Hoechst 33258 dye (Molecular Probe) at room temperature for 10 min, the cells were examined under LEICA DMIL MPS30-inverted phase-contrast fluorescence microscope.

Flow cytometry (FCM)

Cells incubated with FDKL-P70 and via H_2O_2 challenge were harvested by centrifugation. Apoptotic cells were determined by FCM as the method described (Darzynkiewcz et al., 1992). About 1×10^7 cells for each sample were collected, washed with PBS, and fixed in 70% ethanol at 4°C overnight. The cells were then incubated with RNase A (60 μg/ml) at 37°C for 30 min, and then with 50 μg/ml propidium iodide (PI) and NP40 (0.1%) in the dark for 30 min. After removing cellular debris by filtration, the cell suspension was assayed by FCM (Beckton-Dickinson FACSCALIBUR) (Ex 448 nm; Em 620 nm).

Assay of endogenerous antioxidants and lipid peroxide

The measurement of the activity of glutathione peroxidase (GSH-Px), catalase (CAT) and the content of glutathione (GSH) and malondialdehyde (MDA) in the cytoplasm of NG108-15 cells was performed as described previously (Bei et al., 2004). Briefly, to assess antioxidants and lipid peroxide in cell after H_2O_2 injury, the cultures were washed with ice cold PBS and then pooled in 0.1M PBS-0.05 mM EDTA buffered solution and homogenized. The homogenate was centrifuged for 1 h at 10000 × g at 4 °C. The supernatants were used in the measurement. GSH was assayed with GSH Assay Kit. GSH-Px was assessed with GSH-Px Assay Kit by quantifying the rate of oxidation of reduced GSH to oxidized glutathione (GSSG). Catalase activity was assessed with Catalase Assay Kit based on H_2O_2 decomposition. The level of MDA, a product of lipid peroxidation, was measured with MDA Assay Kit based on modified thiobarbituric acid method (Uchiyama and Mihara, 1978). The level of protein in supernatant was determined by the Coomassie blue staining method with bovine serum albumin as the standard as described (Bradford, 1976). All assay kits were from Nanjin Jianchen Biochem Co.

Western blot analysis

Cells incubated with FDKL-P70 and via H_2O_2 challenge were collected for Western immunoblot analysis, which was performed with BM Chromogenic Western blot Kit (Roche Molecular Biochemical). SDS-PAGE and Western blotting were fulfilled by the described method (Sambrook et al., 1989).

Statistical analysis

Except for the Western immunoblot analysis and fluorescence microscopy, all data were shown as the mean ± SEM and statistical significance was evaluated by t-test or one-way ANOVA followed by Duncan's multiple range test where appropriate. $P < 0.05$ was regarded as statistically significant.

Results

Cytotoxicity of H_2O_2 and flavonoids in NG108-15 cells

MTT assays showed that cell viability was strikingly decreased in a concentration-dependant manner after NG108-15 cell cultures were incubated with 200~1000 μM H_2O_2 for 10 hr. The IC_{50} of H_2O_2 in NG108-15 cells is 420.5 μM, indicating that NG108-15 cells were particularly susceptible to H_2O_2-induced cell damage.

At the concentration less than 100 μg/ml, FLDK-P70 showed little effect on cell viability. But when the concentration of FLDK-P70 is higher than 500 μg/ml, cell viability markedly decreased in a concentration-dependent manner (Table 1). The IC_{50} of FLDK-P70 and rutin in NG108-15 cells was 1332.0 μg/ml and 1040.5 μg/ml, respectively.

Protection of flavonoids against H_2O_2 induced injury

MTT assays demonstrated that cell viability was sharply decreased after NG108-15 cell cultures were challenge by 300~500 μM H_2O_2. The leakage of LDH from cytoplasma of cultured NG108-15 cells into medium was dramatically increased by about 188.2%. However, when the cells were

Table 1
Effects of FLDK-P70 and rutin on the cell viability of cultured NG 108-15 cells

Concentration (μg/ml)	FLDK-P70 group Cell viability(%) (OD)	Rutin group Cell viability(%) (OD)
0 (control)	100 ± 7.7(0.325 ± 0.020)	100 ± 6.9(0.332 ± 0.021)
0 (vehicle)	99.8 ± 8.5	
5	102.0 ± 2.5	99.0 ± 3.5
25	103.0 ± 2.8	100.0 ± 2.8
100	101 ± 4.3	100.0 ± 4.1
500	88.0 ± 8.9**	89.0 ± 8.9**
1000	51.0 ± 18.9**	47.0 ± 14.6**
1500	30.7 ± 4.6**	27.8 ± 11.6**
2500	22.2 ± 7.6**	19.0 ± 9.6**

NG108-15 cells were incubated with FLDK-P70 or rutin at different concentrations for 24h, then the cell viability was tested by MTT assay. The data were the means ± SEM shown as the percent of control value, statistical comparison was made using ANOVA followed by Duncan's multiple range test. Three independent experiments were carried out in heptoplicate (n=7).
*, $P < 0.05$.
** $P < 0.01$ *vs* control group.

preincubated with 2~30 μg/ml FLDK-P70 and 2–30 μg/ml (3.28~16.4 μM) rutin, respectively, H_2O_2-induced cell injury was strikingly alleviated in the concentration-dependent way and cell viability was improved. The leakage of LDH from cytoplasma of cultured NG108-15 cells into medium was dramatically restored to normal level. The maximum protection of FLDK-P70 on H_2O_2-induced injury in NG108-15 cells was observed at the concentration 30 μg/ml (Table 2). But less protection was shown when the concentration of FLDK-P70 was higher than 30 μg/ml or that of rutin is higher than 49 μM (30 μg/ml) (Table 2).

MTT assays also showed that the treatment with FLDK-P70 or rutin after H_2O_2-induced injury for 1 hr and 2 hr could attenute the reduction of the viability of NG108-15 cells (Table 3), suggesting that FLDK-P70 or rutin could prevent NG108-15 cell from oxidative stress induced injury although the protection was less than pretreatment.

Inhibition of H_2O_2-induced apoptosis by flavonoids

Hoechst 33258 specifically stains DNA and is widely used to detect shrinkage of the nuclei (chromatin condensation, nuclear fragmentation, appearance of apoptotic bodies are indicative of apoptosis) (Kerr et al., 1995). After NG108-15 cells were treated with H_2O_2 and/or drugs, cells were stained with Hoechst 33258 dye to investigate the nature of H_2O_2-induced cell death.

Typical fluorescence photographs of shrunken nuclei, chromatin condensation and appearance of apoptotic bodies were shown in NG108-15 cell after the exposure to 300 μM H_2O_2 for 10 hr (see Fig.

Table 2
Protection of FLDK-P70 and rutin against NG108-15 cells injury induced by H_2O_2

Group	Concentration (μg/ml)	Cell viability/% (OD) n=7.	Cell apoptosis/% (n=3)	LDH in medium/% ($KU \cdot gpr^{-1}$, n=5)
Control		100 (0.347 ± 0.027)	3.45 ± 0.78	100 ± 7.3 (29.3 ± 2.1)
H_2O_2 model	300 μM	65.6 ± 14.1***	33.07 ± 4.09***	188.2 ± 22.9***
H_2O_2+ FLDK-P70	0(vehicle)	66.1 ± 13.4	32.81 ± 4.37***	186.5 ± 23.2***
	2	80.4 ± 13.5##	31.25 ± 5.28	155.6 ± 23.7##
	10	93.3 ± 7.6##	25.86 ± 4.89##	133.9 ± 27.0##
	30	98.8 ± 4.6##	17.28 ± 4.23##	125.6 ± 23.7##
	50	89.4 ± 3.8##	28.53 ± 8.23##	159.5 ± 41.3
H_2O_2+ Rutin				
	2	91.5 ± 9.8##	32.54 ± 4.50##	160.7 ± 29.9
	10	97.5 ± 3.2##	23.54 ± 4.90##	136.7 ± 26.5##
	30	98.0 ± 3.5##	15.65 ± 4.42##	117.2 ± 36.9##
	50	68.9 ± 13.3	43.54 ± 11.40##	165.8 ± 43.7

NG-108-15 cells were exposed to 300 μM H_2O_2 for 10 hr. FLDK-P70 or rutin was added 2 hr prior to H_2O_2 addition. The cell viability was measured by MTT reduction. The rate of cell apoptosis was measured by FCM. The activity of LDH in the medium of the culture was tested by LDH kit. Data were the mean ± SEM shown as percentages of the corresponding non-H_2O_2 treated control from two to three independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was regarded as statistically significant.

*, $P < 0.05$.; **$P < 0.01$.

*** $P < 0.001$ *vs* non-H_2O_2 teated control group.

#, $P < 0.05$.

$P < 0.01$, *vs* H_2O_2 model group.

Table 3
Protection of postinjury treatment with flavonoids against H_2O_2-induced injury in NG108-15 cells

Group	Concentration (μg/ml)	Treated 1hr after H_2O_2 challenge Cell viability/% (OD) n=7	Treated 2hr after H_2O_2 challenge Cell viability/% (OD) n=7
Control		100 (0.321 ± 0.023)	100 (0.326 ± 0.028)
H_2O_2 model	300 μM	65.6 ± 8.1***	64.8 ± 11.5***
300 μM H_2O_2+ flavonoids			
vehicle		66.1 ± 7.2.	65.3 ± 7.2
FLDK-P70	2	73.0 ± 8.3$^{\#}$	70.5 ± 8.1
FLDK-P70	10	85.3 ± 6.5$^{\#\#}$	76.5 ± 3.9$^{\#}$
FLDK-P70	30	91.8 ± 4.9$^{\#\#}$	82.0 ± 3.6$^{\#\#}$
FLDK-P70	50	77.5 ± 8.6$^{\#\#}$	72.9 ± 11.8
Rutin	2	71.5 ± 9.1$^{\#}$	68.5 ± 7.9
Rutin	10	84.5 ± 3.5$^{\#\#}$	76.1 ± 3.8$^{\#\#}$
Rutin	30	88.0 ± 3.3$^{\#\#}$	81.3 ± 3.8$^{\#\#}$
Rutin	50	72.3 ± 11.8	68.9 ± 14.8

NG-108-15 cells were exposed to 300 μM H_2O_2 for 10 hr. FLDK-P70 or rutin was added 1 or 2 hr after H_2O_2 challenge. The cell viability was measured by MTT reduction. Data were the mean ± SEM shown as percentages of the corresponding non-H_2O_2 treated control from two to three independent experiments. Statistical significance was evaluated by one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was regarded as statistically significant.
*, $P < 0.05$; **$P < 0.01$.
*** $P < 0.001$ *vs* non-H_2O_2 teated control group.
$^{\#}$ $P < 0.05$.
$^{\#\#}$ $P < 0.01$, *vs* H_2O_2 model group.

1A, Fig. 2B and Table 2), which strongly suggest that apoptosis was induced in NG108-15 cells by the challenge of 300 μM H_2O_2. Assay by FCM showed that approximately 33% cells committed apoptosis after exposure to 300 μM H_2O_2 (see Fig. 2B and Table 2).

However, preincubation of the cells with 2~30 μg/ml FLDK-P70 or rutin for 2 hr was potent to reduce the percentage of H_2O_2-induced apoptotic cells (Fig. 1B-F, Fig. 2A, C-F and Table 2). But less protection was shown when the concentration of FLDK-P70 or rutin was higher than 30 μg/ml (Table 2).

Flavonoids improves the redox equilibrium

It was generally known that superoxide dismutase (SOD), GSH-Px and catalase are the key antioxidant enzymes and GSH is the main antioxidant and MDA is the main index for lipid peroxidation in cells. After NG108-15 cells were challenged by 300 μM H_2O_2, GSH-Px, catalase activities and the GSH content were sharply decreased. The reduction induced by H_2O_2 in the activities of GSH-Px and catalase was 47.8% and 65.2%, respectively, but the MDA level was increased by 169% (Table 4). In contrast, the activity of SOD in NG108-15 cells had little change (data not show). However, the pretreatment with FLDK-P70 strikingly alleviated the alternation in GSH-Px, catalase activities and GSH and MDA level in H_2O_2-challenged NG108-15 cells. Moreover, when cells were pretreated with 10 or 30 μg/ml FLDK-P70, GSH returned to normal control level and even a little higher (Table 4). Similarly, rutin alleviated H_2O_2-induced redox desequilibrium in the same fashion.

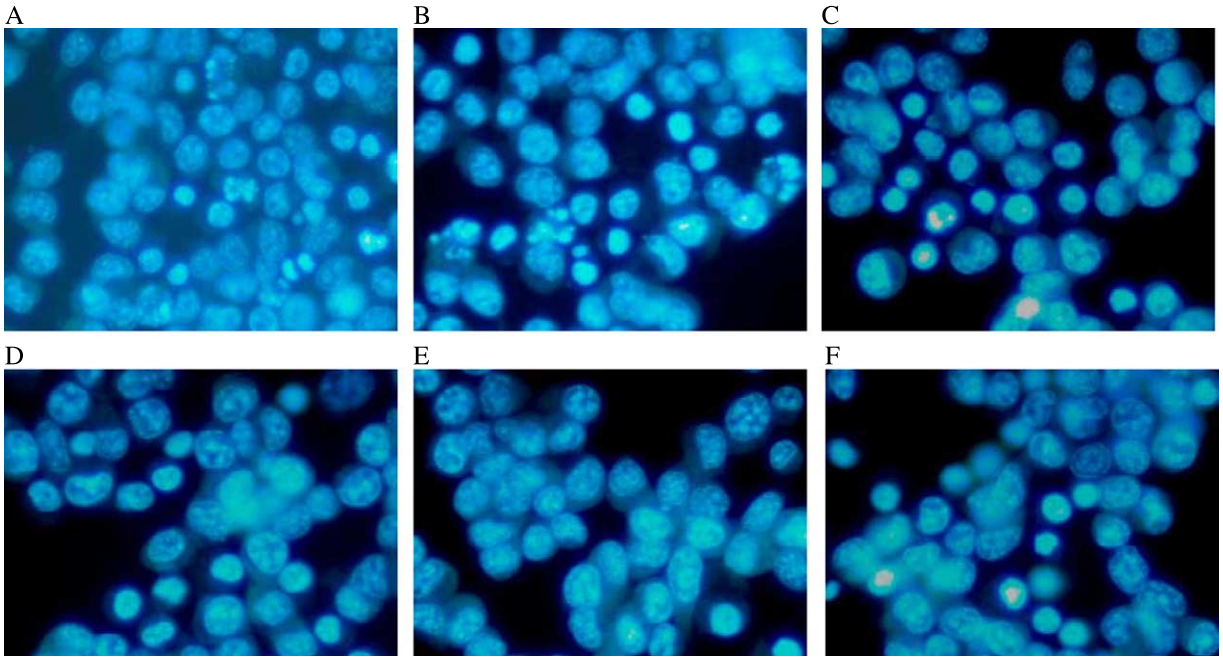

Fig. 1. Protection of FLDK-P70 against apoptosis induced by H_2O_2 in NG108-15 cells. Cells were incubated with 300 μM H_2O_2 for 10 hr. Flavonoids were added to the culture 2 hr prior to H_2O_2 addition. Cells were harvested, fixed in paraformaldehyde and examined by fluorescence photomicrographs stained with Hoechst 33258 dye. (A): NG108-15 cells exposed to 300 μM H_2O_2; (B): NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 2 μg/ml FLDK-P70; (C): NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 10 μg/ml FLDK-P70; (D): NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 30 μg/ml FLDK-P70; (E): Control NG108-15 cells; (F): NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 10 μg/ml (16.4 μM) rutin. Pictures were taken using an LEICA DMIL MPS30 inverted phase-contrast microscope(×400). The figures were the example of three separate tests with similar results. Hoechst 33258 dye was used to stain the DNA of the shrunken nuclei and the chromatin condensation in nuclei in the fluorescence pictures. (A) shows typical example of fluorescence microscopy pictures of NG108-15 cells in apoptosis, indicating that the chromatin condensation in nuclei and appearance of apoptotic bodies was induced with 300μM H_2O_2 for 10 hr.

FLDK-P70 upregulated Bcl-2 expression

Western blotting analysis showed that the pretreatment with 10–30 μg/ml FLDK P70 or 10 μg/ml rutin could significantly increase *bcl-2* expression in NG108-15 cells exposed to 300 μM H_2O_2 for 10 hr (Fig. 3).

Discussion

We have recently shown that NXQ exhibited significant neuroprotection against H_2O_2 induced injury (Bei et al., 2004). In the present study, a series of experiments designed to investigate the effects of FLDK-P70, which is one of the main components of NXQ. NG108-15 cells exposed to 300 μM H_2O_2 showed significantly oxidative stress injury, by which, cell viability was markedly decreased and the activities of CAT, GSH-Px and the content of GSH were reduced significantly, but the content

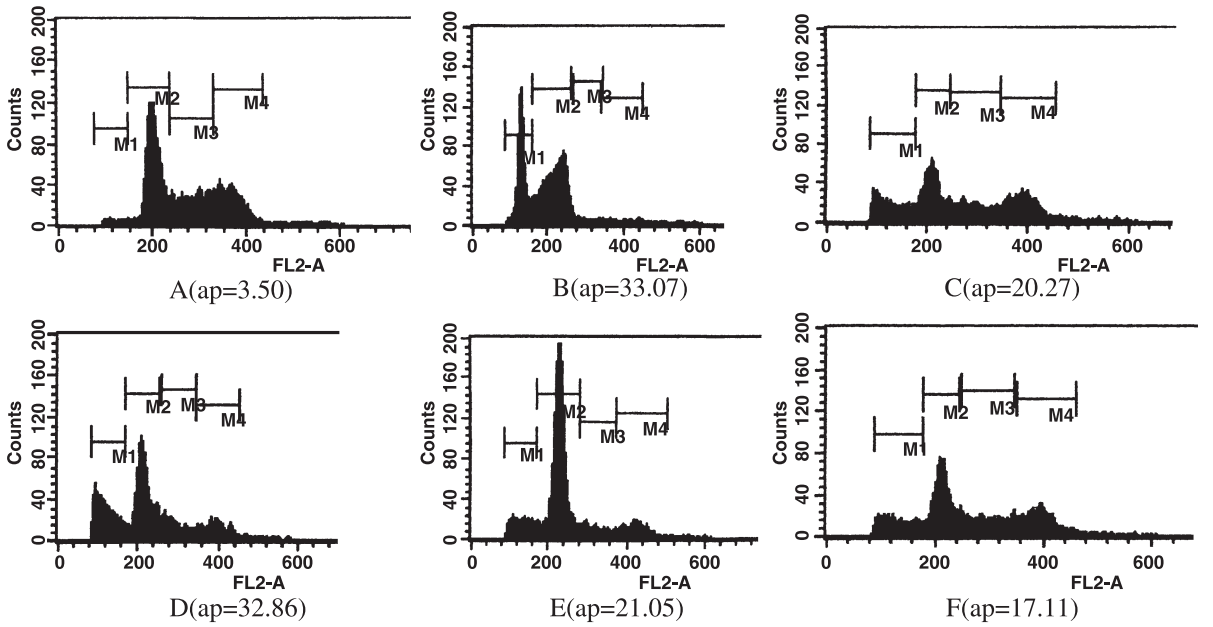

Fig. 2. Protection of FLDK-P70 against H_2O_2-induced apoptosis in NG108-15 cells assessed by FCM. Cells were exposed to 300 μM H_2O_2 for 10 hr. FLDK-P70 was added to the culture 2 hr before H_2O_2 addition. Cells then were harvested and determined by FCM. The peaks of M1 represented cells in G_0-G_1 phase subgroup. The presence of hypodiploid peak (M1) reflects the number of cells suffering from DNA fragmentation. Apoptotic rate (%) is designated as 'ap'. (A) Control NG108-15 cells; (B) NG108-15 cells challenged with 300 μM H_2O_2. (C) NG108-15 cells challenged with 300 μM H_2O_2 in the presence of 10μg/ml (16.4 μM) Rutin. (D) NG108-15 cells challenged with 300 μM H_2O_2 in the presence of 2 μg/ml FLDK-P70. (E) NG108-15 cells challenged with 300 μM H_2O_2 in the presence of 10 μg/ml FLDK-P70. (F) NG108-15 cells challenged with 300 μM H_2O_2 in the presence of 30 μg/ml FLDK-P70. The figure was the example of three separate test with similar results.

of MDA and the release of LDH from cell to medium increased sharply indicating that oxidative injury appeared in NG108-15 cells. The observation is well coherent to the pathological change of the brain neurons in vivo under oxidative stress during ischemia or ischemia/reperfusion injury (Ram Gupta et al., 2003).

The preincubation with 2~30 μg/ml FLDK-P70 alleviated H_2O_2-induced oxidative stress injury by improving redox balance in the concentration-dependent fashion. Reducing the elevation of MDA of cells and the release of intracellular LDH into culture medium and maintaining the activity of intracellular antioxidant enzymes: CAT, GSH-Px and the content of the intracellular antioxidant GSH, confirmed the efficacy of anti-oxidation of FLDK-P70 in NG108-15 cells. The treatment with 2~30 μg/ml FLDK-P70 or rutin after H_2O_2-induced injury also alleviated H_2O_2-induced oxidative stress injury. These results suggested that FLDK-P70 has a potential protection of NG108-15 cells from oxidative stress injury. Rutin, a common flavonoid glucoside of quercetin showed similar effects.

Quercetin and kaempfetol were discovered to be the main aglycones of FLDK-P70. Besides, the glucosides of quercetin and kaempfetol, such as rutin, astragalin, hyperin and isoquercitrin were also found in FLDK-P70 data not showed. These flavonoids containing phenolyl show very potent antioxidant effects (Afanas'ev et al., 1989; Ishige et al., 2001), which might contribute to the protection of FLDK-P70 on NG108-15 cells from oxidative stress injury.

Table 4
Effect of FLDK-P70 on the intracellular antioxidant of NG108-15 cells under H_2O_2 challenge

Group	Concentration/μg/ml	GSH (mg/gprot)	CAT (U/mgprot)	GSH-Px (U/mgprot)	MDA (nmol/mgprot)
Control	control	100 ± 12.5 (123.9 ± 15.6)	100 ± 7.9 (4.42 ± 0.56)	100 ± 9.8 (68.80 ± 6.76)	100 ± 11.2 (1.43 ± 0.16)
H_2O_2 Model	300 μM	72.2 ± 11.28**	34.8 ± 12.5**	52.2 ± 8.3**	169.2 ± 18.6**
H_2O_2 +					
FLDK-P70	2	85.9 ± 14.0$^{\#}$	52.9 ± 13.9$^{\#}$	61.3 ± 8.5$^{\#}$	145.1 ± 17.9$^{\#}$
FLDK-P70	10	107.27 ± 7.94$^{\#\#}$	67.0 ± 15.7$^{\#\#}$	69.0 ± 12.1$^{\#\#}$	130.0 ± 17.6$^{\#\#}$
FLDK-P70	30	130.6 ± 20.3$^{\#\#}$	88.6 ± 14.9$^{\#\#}$	89.5 ± 19.6$^{\#\#}$	123.6 ± 18.3$^{\#\#}$
Rutin	10	109.9 ± 39.5$^{\#\#}$	74.0 ± 31.0$^{\#\#}$	72.0 ± 11.9$^{\#\#}$	135.0 ± 16.4$^{\#\#}$
Rutin	30	120.7 ± 33.3$^{\#\#}$	86.2 ± 18.7$^{\#\#}$	83.2 ± 18.3$^{\#\#}$	119.6 ± 15.8$^{\#\#}$

Cultured NG108-15 cells were treated with FLDK-P70 or rutin at different concentrations for 2 hr prior to exposure to 300 μM H_2O_2 for 10 hr, then the activity of CAT, GSH-PX and the contents of GSH, MDA in the cytoplasm were tested by corresponding kits. Data were the mean ± SEM expressed as percentages of the corresponding non-H_2O_2 teated control from two to three independent experiments (n = 5–6). Statistical significance was evaluated by one-way ANOVA followed by Duncan's multiple range test. $P < 0.05$ was regarded as statistically significant.
*, $P < 0.05$.
** $P < 0.01$ *vs* non-H_2O_2 teated control group.
$^{\#}$ $P < 0.05$.
$^{\#\#}$ $P < 0.01$, *vs* H_2O_2 model group.

It is the first time to demonstrate that FLDK-P70 improved the equilibrium of redox and inhibited the apoptosis and increased the cell viability in NG108-15 cells under oxidative stress induced by H_2O_2.

Oxidative stress and mitochondrial dysfunction play a key role in the apoptotic event. Apoptotic damage will make the brain more susceptible to further pathology (Xiao et al., 2000). Although the molecular mechanisms of how FLDK-P70 protects neurons against oxydative stress remain to be fully understood, by alleviating oxidative damage in NG108-15 cells, FLDK-P70 might not only delay the neurodegenerative process, but also help the recovery of neuronal function.

GSH, GSH-Px, catalase, and SOD, along with GSH and other non-enzymatic antioxidants constitute defence mechanism of cells to protect cell from the damage caused by ROS. Elevation in levels or activation of endogenous antioxidant enzymes resulted in prevention of oxidative injuries (Yassi et al., 2002). Our present studies confirmed that NG108-15 cells challenged by H_2O_2 caused a striking decrease in cell viability with LDH release into culture medium and redox desequilibrium with character of an accumulation of MDA, and reduction of activities of GSH-Px, and catalase as well as low level of GSH in cells (Tanaka et al., 2001). Moreover, we also showed that intermediate concentrations of H_2O_2 induced apoptosis in NG108-15 cells.

However, when NG108-15 cells were preincubated with FLDK-P70 or rutin, the H_2O_2-induced reduction of activities of catalase and GSH-Px and the level of GSH was evidently alleviated as the H_2O_2-induced cell death reduced. H_2O_2-induced apoptosis was also strikingly alleviated, suggesting that cytoprotective effects of FLDK-P70 or rutin were due to lessening the consumption of antioxidant enzymes caused by ROS.

GSH is the key endogenous antioxidant. The content of GSH in cell is important in regulation of apoptosis (Hall, 1999; Mizui et al., 1992). Maintaining of the content of GSH by FLDK-P70 might play a key role in reducing apoptosis in NG108-15 cells under oxidative stress. This is in accord with the

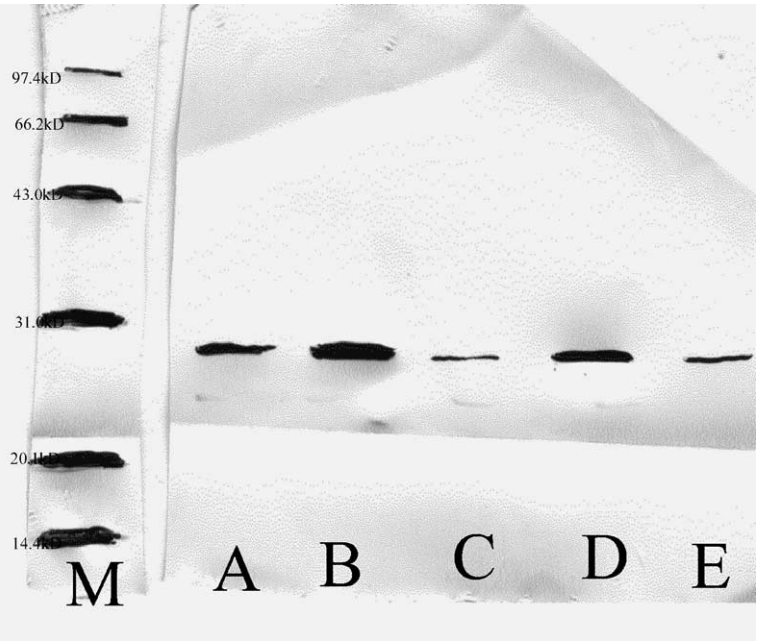

Fig. 3. Upregulation of bcl-2 expression in NG108-15 cells by FLDK-P70 under oxidative stress. Western blot analysis of expression of bcl-2 were carried out after NG108-15 cells exposed to 300 μM H_2O_2 for 10 hr. M: Protein marker, A: NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 10 μg/ml FLDK-P70; (B) NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 30 μg/ml FLDK-P70; (C) Control NG108-15 cells; (D) NG108-15 cells exposed to 300 μM H_2O_2 in the presence of 10 μg/ml (16.4 μM) rutin; (E) NG108-15 cells exposed to 300 μM H_2O_2. The figure was the example of two seperate experiments with similar results.

previous report that flavonoids inhibited the decrease of GSH, and maintained the activities of CAT, GSH-px and GSH in neuronal cells under the glutamate toxicity to protect the neurons (Ishige et al., 2001).

Free radicals are involved in cerebral ischemia and reperfusion-induced neuronal injury (Yassi et al., 2002). It was reported that flavonoids scavenge free radicals (Robak and Gryglewski, 1998) and that rutin and quercetin scavenge superoxide anions (Afanas'ev et al., 1989). Futhermore, rutin also inhibited Fenton reaction by chelating Fe2+ ion to reduce the generation of free radical (Afanas'ev et al., 1989). Therefore, the striking neuroprotective effects of FLDK-P70 or rutin against H_2O_2-induced injury in the present study might be attributed to its ability to scavenge free radical and prevent their formation. This notion is supported by the study in which rutin strikingly prevented global cerebral ischemia and reperfusion induced elevation in mitochondrial MDA generation at the same dose levels at which it decreased cerebral infarct size in rat (Ram Gupta et al., 2003).

Although the physiological benefits of flavonoids have been largely ascribed to their antioxidant properties in plasma, flavonoids may also protect cells from various challenges. Three protective mechanisms were reported for flavonoids in a cell culture model of oxidative stress as followed: 1) increasing intracellular GSH, 2) directly lowering levels of ROS, 3) preventing the influx of Ca^{2+} despite of high levels of ROS (Ishige et al., 2001). The protection from oxidative challenges by flavanoids may

have its highly specific machenism for each of their compounds. Flavonoids also showed protective efficacy in many animal models of oxidative stress.

Bcl-2 is a key anti-apoptosis gene and a mitochondrial protein involved in delayed neuron death and cerebral ischemic injury and protect neurons from apoptotic stimuli including oxidative stress. Bcl-2 expression protects neurons from cerebral ischemia damage by inhibiting the formation of reactive oxygen molecules and by increasing ischemia tolerance and resistances to oxidative stress of neuron and by scavenging free radical directly (Kane et al., 1993; Martinou et al., 1994). Bcl-2 can also maintain intracellular Ca^{2+} hemeostasis and inhibite the release Ca^{2+} from endoplasma, and inhibits the apoptosis induced by *Fas,* P_{53} and ICE (Chen and Simon, 1997).

FLDK-P70 and rutin could up-regulate *bcl-2* gene expression in NG108-15 cells exposed to H_2O_2. This is coherent with the previous report (Shahed et al., 2001). It may be one of the important mechanisms to protect neurons from oxidative stress injury.

In addition, the present study showed that highly purified flavonoids are directly toxic to cells, though the exact mechanism involved remained unclear. The present study showed that high concentration of flavonoid (>50–100 μM) reduced their neuroprotection from oxidative stress injury in NG108-15 cells, like the previous report (Nagao et al., 1999). This might contribute to their activities to produce free radical to induce apoptosis. It was proposed that the addition of reducing agents to commonly used cell-culture media could lead to generation of substantial amounts of H_2O_2 (Halliwell et al., 2000). Some of the reported effects of ascorbic acid and polyphenolic compounds (e.g., quercetin, etc) on cells in culture may be due to H_2O_2 generation by interaction of these compounds with cell culture media (Halliwell et al., 2000). This might partly explain why excess highly purified flavonoid showed little protection to NG108-15 cells when exposed to H_2O_2, though flavnoids reduce oxidative injury by scavenging free radicals.

Conclusion

Our study demonstrated that FLDK-P70 protected nervous cell from the injury of oxidative stress by upregulating *bcl-2* and improving cellular redox state. FLDK-P70 might be the key ingredient of NXQ and be potentially used in the prevention and treatment of ischemia/reperfusion injury disorder and other neurodegenerative disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lfs.2004.09.031.

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