Protective effects of naringenin-7-*O*-glucoside on doxorubicin-induced apoptosis in H9C2 cells

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Abstract

Doxorubicin, a widely used chemotherapeutic agent, can give rise to severe cardiotoxicity by inducing cardiomyocyte apoptosis. *Dracocephalum rupestre* Hance, a Chinese traditional herb, has therapeutic potential for cardiovascular diseases. Naringenin-7-*O*-glucoside is the main active constituent of *D. rupestre* and there is increasing interest in its therapeutic applications. The aim of this study was to evaluate the effects of naringenin-7-*O*-glucoside on cardiomyocyte apoptosis induced by doxorubicin. Cell viability was detected by MTT assay. Naringenin-7-*O*-glucoside (10, 20, and 40 μM) significantly enhanced cardiomyocyte proliferation relative to that of doxorubicin. Furthermore, naringenin-7-*O*-glucoside increased the protein levels of heme oxygenase-1 (HO-1) and Bcl-2 in cardiomyocytes (as detected by Western blotting) and suppressed the mRNA expression of caspase-3 and caspase-9 (as detected by RT-PCR). These results suggest that naringenin-7-*O*-glucoside has protective effects against doxorubicin-induced apoptosis, effects which could underlie the use of naringenin-7-*O*-glucoside therapeutic agent for treating or preventing cardiomyopathy associated with doxorubicin.
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Keywords: Naringenin-7-*O*-glucoside; Heme oxygenase-1; Bcl-2; Apoptosis; Doxorubicin

1. Introduction

Doxorubicin is an anthracycline antibiotic with a broad spectrum of activity and high potency against human malignant neoplasms. However, its clinical use is limited by its severe cumulative dose-related cardiotoxicity, which leads to the development of cardiomyopathy (Shan et al., 1996; Gorelik et al., 2003) and heart failure (Keefe, 2001). It has been demonstrated that the development of doxubicin cardiomyopathy involves apoptosis of cardiomyocytes (L'Ecuyer et al., 2006; Takemura and Fujiwara, 2007). Since cardiomyocyte apoptosis contributes to the development of myocardial dysfunction in heart failure, inhibition of doxorubicin-induced cardiomyocyte apoptosis may provide new opportunities for the prevention and treatment of doxorubicin-induced heart failure (Christiansen and Autschbach, 2006).

Flavonoids, a group of polyphenols, possess potent cardioprotective efficacy and significantly reduce the risk of cardiovascular disease (Bast et al., 2007; Du et al., 2007; Peluso, 2006; Huxley and Neil, 2003). Some research groups have reported that flavonoids exhibit protective effects against cardiomyopathy and cardiomyocyte apoptosis induced by doxorubicin (Bagchi et al., 2003; Hüsken et al., 1995). The Chinese traditional medicine *Dracocephalum rupestre* Hance, a wild perennial herb found throughout western China, is a rich resource of flavonoids (Wu and Li, 1977). Therefore, it has therapeutic potential for cardiovascular diseases. In our continuous search for cardioprotective substances from natural products, naringenin-7-*O*-glucoside, a major active flavonoid isolated from *D. rupestre* Hance, has attracted our interest as a compound potentially able to prevent cardiomyocyte apoptosis induced by doxorubicin.

In the present study, we investigated the effects of naringenin-7-*O*-glucoside on cardiomyocyte apoptosis induced by doxorubicin. The results demonstrated that naringenin-7-*O*-glucoside was able to attenuate doxorubicin-induced H9C2 cell apoptosis, having an effect comparable to that of quercetin.

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2. Materials and methods

2.1. Chemicals and materials

Naringenin-7-*O*-glucoside was isolated from *D. rupestre* Hance in our laboratory (Ren et al., 2003; Ren et al., 2005) and dissolved in dimethylsulfoxide (DMSO) for the in vitro bioassay.

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was bought from Tianjin TBD Biotechnology Development Center (Tianjing, China). Pepstatin A, Hoechst 33258, phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), and 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2-tetrazolium bromide (MTT) were purchased from Sigma. Dithiotreitol (DTT) was bought from Merck. Annexin V-FITC kit was purchased from Jingmei Biotech (Shenzhen, China). Diethylpyrocarbonate (DEPC), acrylamide, RNase inhibitor, and leupeptin were bought from Amresco. 3,3′-Diaminobenzidine tetrahydrochloride substrate kit (DAB) was bought from Zhongshan Goldenbridge Biotechnology LTD (Beijing, China). Polyvinylidene difluoride (PVDF) was bought from Millipot. RNA PCR kit was bought from TaKaRa. An antibody against HO-1 was purchased from Chemicon. Antibodies against Bcl-2, β-actin and horseradish peroxidase-conjugated secondary antibody were obtained from Boster Biological Technology LTD (Wuhan, China).

2.2. Cell culture

Rat cardiac H9C2 cells (ATCC Rockville, MD) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 5% CO_2 at 37 °C. The cells were fed every 2–3 days and subcultured once they reached 70–80% confluence. Cells were plated at an appropriate density according to each experimental design.

2.3. MTT assay

A modified MTT assay was used to determine cell viability (Cao and Li, 2004). The H9C2 cells were incubated with chemicals in DMEM supplemented with 0.5% fetal bovine serum at 37 °C for 24 h, and then treated with MTT solution (final concentration, 0.5 mg/ml) for 4 h at 37 °C in 96-well plates. The supernatants were removed carefully, followed by the addition of 100 μl DMSO to each well to dissolve the precipitate. Then, the absorbance was measured at 570 nm in a microplate reader (Synergy HT).

2.4. Fluorescent staining of nuclei with H33258

The nuclei of H9C2 cells were stained with chromatin dye (Hoechst 33258) (Du et al., 2007). The cells were fixed with 3.7% paraformadehyde for 10 min, washed twice with PBS, and incubated with 10 μM Hoechst 33258 in PBS at room temperature for 30 min. After three washes, the cells were observed under a fluorescence microscope (IX-7, Olympus, Japan).

2.5. DNA fragmentation assay

The DNA fragmentation assay was performed according to the method of Herrmann et al. (1994). Briefly, after being harvested, the cell samples were washed with PBS and pelleted by centrifugation. The cell pellets were then treated for 10 s with lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH 7.5). After centrifugation for 5 min at 1600 ×g, the supernatant was collected and the extraction was repeated with the same amount of lysis buffer.

The supernatants (and resuspended nuclei as control for the complete recovery of the apoptotic DNA fragments) were added to 1% sodium dodecyl sulphate (SDS) and treated for 2 h with RNase inhibitor (final concentration 5 μg/μl) at 56 °C, followed by digestion with proteinase K (final concentration 2.5 μg/μl) for at least 2 h at 37 °C. After addition of 1/2 vol. 10 M ammonium acetate, DNA was precipitated with 2.5 vol. ethanol, dissolved in gel loading buffer, and separated by electrophoresis in 2% agarose gels containing 0.1 μg of ethidium bromide, and visualized under UV light.

2.6. Flow cytometric detection of apoptosis

FITC-Annexin V/propidium iodide (PI) double staining was performed as follows (Spallarossa et al., 2005). After being washed twice with PBS, cells (1×10^6) were resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), and FITC-Annexin V and PI, at concentrations of 1 μg/ml, respectively, were added. The mixture was incubated for 10 min in the dark at room temperature, and then cellular fluorescence was measured by flow cytometry analysis with a FACS-SCAN apparatus (FACSCalibur; BD Biosciences, USA).

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from H9C2 cells using Trizol (Invitrogen). First-strand cDNAs were generated by reverse transcription using oligo (dT) from RNA samples. Primer sequences (Sbsgene, Shanghai, China) are shown below. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LP: 5′-GAGGGGCCATC-CACAGTCTTCTG-3′, RP: 5′-CCCTTCATTGACCTCAACTA-CATGGT-3′; HO-1, LP: 5′-AGCGAAACAAGCAGAACCCA-3′, RP: 5′-GATGCTCGGGAAGGTGAAAA-3′; Caspase-9, LP: 5′-CCCCACCCTCACTTTGCT-3′, RP: 5′-TGGAGGACCAGGCT-CACTTA-3′; Caspase-3, LP: 5′-AGGCTACCATCCTT-CAGTG-3′, RP: 5′-TCTCCTTTCCTTACGCTCT-3′; Bcl-2, LP: 5′-GACGCGAAGTGCTATTGGT-3′, RP: 5′-TCAGGCTGGAAGGAGAAGAT-3′. After cDNA synthesis, PCR was performed, using the following conditions: 95 °C for 3 min; 94 °C for 30 s; 58 or 60 °C for 30 s (depending on the sequences of the primers); and 72 °C for 1 min, 72 °C for 5 min for 30 cycles. PCR products were electrophoresed in 2% agarose gel and visualized with ethidium bromide (EB). The relative expression was quantified densitometrically using the AlphaImager™ 2200 System (Alpha Innotech

Corporation) and calculated according to the reference bands of GAPDH.

2.8. Western blot analysis

Treated cells (1×10^7 cells) were collected and washed with ice-cold PBS and resuspended in 75 µl of RIPA buffer (50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% Triton-X 100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 60 mM DTT, 1 mM PMSF, 2 µg/ml leupeptin, 5 µg/ml pepstatin A). Cell lysis was carried out at 4 °C by vortexing for 15 s, and the cell suspension was then stored for 10 min in ice. After centrifugation at 13,000 ×g for 10 min, the supernatant was separated and stored at −70 °C until use.

The protein concentration was determined using the Bradford protein assay kit (Beyotime Institute of Biotechnology, Beijing, China). After addition of sample loading buffer, protein samples were electrophoresed on a 15% SDS–polyacrylamide gel. Proteins (25 µg) were transferred to PVDF blots at 150 mA for 2 h. The blots were blocked for 2 h at room temperature in PBST (PBS and 0.1% Tween 20, pH 7.4, containing 5% non-fat dried milk). Sheets were incubated with rabbit anti-HO-1 antibody (1:500), rabbit anti-Bcl-2 antibody (1:200) and rabbit anti-beta-actin antibody (1:200) in PBST with 5% non-fat dried milk. Following three washes with PBST, the blots were incubated with horseradish peroxidase-conjugated goat antirabbit monoclonal antibody (1:2000) for beta-actin and HO-1 or Bcl-2 in PBST with 5% non-fat dried milk for 2 h at room temperature. Then sheets were washed again three times in PBST buffer, and transferred proteins were incubated and visualized with DAB substrate solution for 10 min, according to the manufacturer's instructions.

2.9. Statistical analysis

All data are expressed as means±S.E.M. from at least three independent experiments. Differences between mean values of multiple groups were analyzed by Student's t-test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Naringenin-7-O-glucoside protects H9C2 cells from doxorubicin-induced cytotoxicity

H9C2 cells were treated with naringenin-7-O-glucoside (10, 20, 40, and 80 µM) in the absence of doxorubicin for 24 h and then the rates of cell growth inhibition were evaluated based on the OD value as estimated with the MTT assay. As shown in Fig. 1A, naringenin-7-O-glucoside at each of these concentrations alone did not cause any apparent cytotoxicity.

To analyze the protective effects of naringenin-7-O-glucoside on doxorubicin-induced cytotoxicity in H9C2 cells, cell proliferation was examined after incubation with naringenin-7-O-glucoside in the presence of doxorubicin. As shown in Fig. 1B, naringenin-7-O-glucoside (10, 20, 40 µM) pretreatment had a significant protective effect
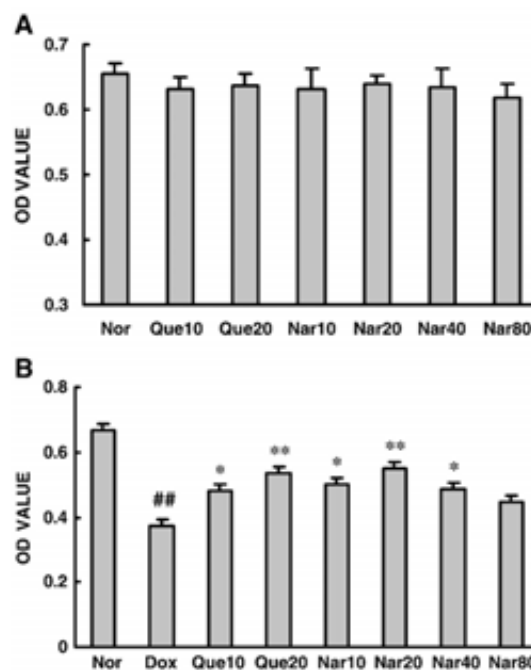

Fig. 1. Protective effects of naringenin-7-O-glucoside pretreatment on doxorubicin-mediated cytotoxicity in H9C2 cells. Proliferation of H9C2 cells exposed to naringenin-7-O-glucoside in the absence (A) or presence (B) of doxorubicin in vitro. Cells were incubated without or with naringenin-7-O-glucoside (10, 20, 40, 80 µM) and quercetin (10, 20 µM) for 24 h, followed by incubation with (20 µM) doxorubicin for another 24 h. After this incubation, cell viability was determined with the MTT assay. Nor: normal cells; Dox: doxorubicin; Nar: naringenin-7-O-glucoside; Que: quercetin. Values represented means±S.E.M. (n=3). #Significantly different from normal; *Significantly different from doxorubicin.

against doxorubicin-mediated cytotoxicity in a dose-dependent manner at low doses. The protective effect decreased at doses higher than 80 µM.

3.2. Effects of naringenin-7-O-glucoside on doxorubicin-induced apoptosis in H9C2 cells

To determine the effects of naringenin-7-O-glucoside on apoptosis induced by doxorubicin in H9C2 cells, we examined cell morphology after Hoechst 33258 staining. As shown in Fig. 2, doxorubicin induced rapid changes in the nuclear morphology of H9C2 cells, with heterogeneous intensity and chromatin condensation being apparent under fluorescence microscope. Normal cells were seen as round-shaped nuclei with a homogeneous fluorescence intensity. Pretreatment with 10, 20, and 40 µM naringenin-7-O-glucoside significantly protected the cells from the morphological changes induced by doxorubicin. The effect decreased at doses higher than 80 µM.

Then, we analyzed chromosomal DNA fragmentation by agarose gel electrophoresis. As shown in Fig. 3, chromatin of H9C2 cells underwent internucleosomal cleavage after treatment with doxorubicin, as demonstrated by the formation of characteristic ladder pattern of DNA migration. Naringenin-7-O-glucoside (10, 20, and 40 µM) pretreatment significantly prevented DNA fragmentation, further confirmed by flow cytometric detection. As shown in Fig. 4, naringenin-7-O-
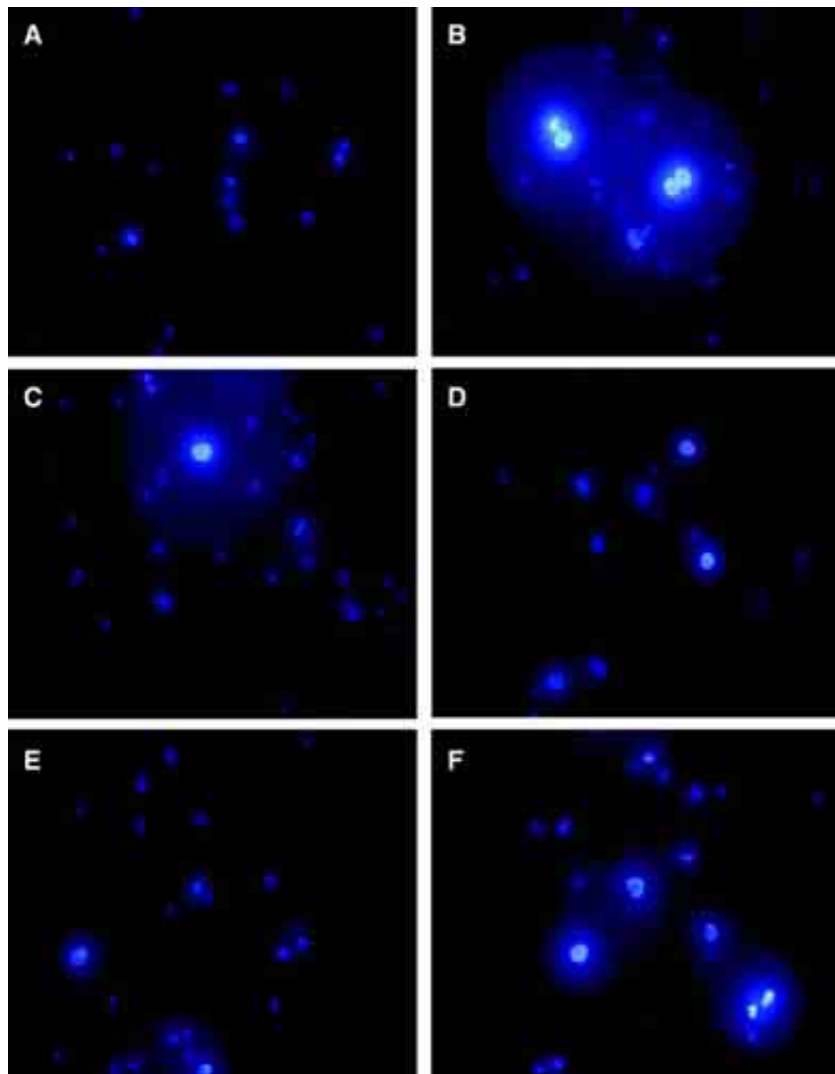
Fig. 2. Morphology of apoptotic H9C2 cells. H9C2 cells were treated without (A) or with 10 (C), 20 (D), 40 (E) and 80 μM (F) naringenin-7-O-glucoside for 24 h, followed by incubation with 10 μM doxorubicin (B) for another 24 h. The cells were then subjected to H33258 staining and viewed under a fluorescence microscope (magnification, ×400).

glucoside (10, 20, and 40 μM) pretreatment provided a significant protective effect against doxorubicin-mediated apoptosis.

3.3. Effects of naringenin-7-O-glucoside on the mRNA expression of caspase-3, caspase-9, Bcl-2, and HO-1

To investigate the mechanisms by which naringenin-7-O-glucoside prevented doxorubicin-induced apoptosis in H9C2 cells, we investigated the mRNA expression of caspase-3, caspase-9, Bcl-2, and HO-1. As shown in Fig. 5, the addition of doxorubicin to the cells induced a drop in the mRNA expression of Bcl-2 and an increase in the expression of caspase-3 and caspase-9. Naringenin-7-O-glucoside pretreatment (10, 20 μM) significantly prevented the decrease in Bcl-2 expression and the increase in caspase-3 and caspase-9 mRNA expression. Furthermore, doxorubicin decreased the expression of HO-1 mRNA in cardiomyocytes. Naringenin-7-O-glucoside (10, 20, and 40 μM) increased the expression of HO-1 mRNA significantly.

3.4. Effects of naringenin-7-O-glucoside on the protein level of Bcl-2, and HO-1

Western blot analysis was performed to explore the effects of naringenin-7-O-glucoside on the protein level of HO-1 and Bcl-2 in H9C2 cells. As shown in Fig. 6, naringenin-7-O-glucoside (10, 20, and 40 μM) pretreatment significantly upregulated the protein level of HO-1 and Bcl-2.

4. Discussion

Although doxorubicin is an effective anticancer chemotherapeutic agent, its clinical use is limited by cumulative dose-related cardiotoxicity, which may lead to severe and irreversible cardiomyopathy (Gewirtz, 1999; Kim et al., 2006a) and heart failure (Keefe, 2001). A proposed mechanism of cardiotoxicity of doxorubicin is that it causes cardiomyocyte apoptosis. Studies have shown that the apoptosis of cardiomyocytes, as characterized by caspase activation, caspase-mediated proteolytic
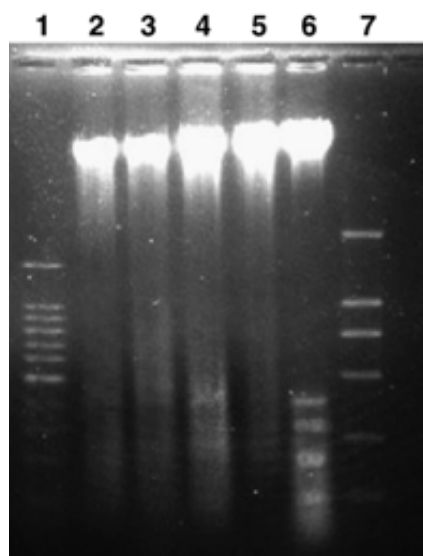
Fig. 3. DNA laddering assays. Gel electrophoresis of DNA extracted from H9C2 exposed to doxorubicin (10 μM) plus naringenin-7-O-glucoside (10, 20, 40 μM) for 24 h. Lane 1 and lane 7: the molecular weight marker; Lane 6: doxorubicin; Lane 5: normal cells; Lane 4: naringenin-7-O-glucosid 40 μM; Lane 3: naringenin-7-O-glucosid 20 μM; Lane 2: naringenin-7-O-glucosid 10 μM.

degradation, and internucleosomal DNA cleavage, contributes to the development of myocardial dysfunction in heart failure (Maejima et al., 2005). Doxorubicin-induced apoptosis is mediated by a decreased the expression of Bcl-2 and an increased the expression of caspase-3 in cardiomyocytes (Wu et al., 2002; Bagchi et al., 2003; Takemura and Fujiwara, 2007). Bcl-2 is known as a critical inhibitor of apoptosis and prevents the release from mitochondria into the cytosol of apoptogenic factors, including cytochrome *c* and apoptosis-inducing factor. Bcl-2 overexpression has been shown to block cell death. Caspase-3 activation is a key step in apoptosis. Accordingly, inhibition of doxorubicin-induced cardiomyocyte apoptosis by modulation of the factors that control apoptosis may provide new opportunities for the prevention and treatment of doxorubicin-induced cardiomyopathy (Christiansen and Autschbach, 2006; Narula et al., 1999; Takemura and Fujiwara, 2007).

Naringenin-7-O-glucoside is a major bioactive constituent of *D. rupestre* Hance. In this study, we first investigated its protective effect against doxorubicin-induced cardiotoxicity. The results showed that treating cells with doxorubicin resulted in cell viability loss. However, pretreatment with different concentrations (10, 20, 40 μM) of naringenin-7-O-glucoside greatly decreased the loss of cell viability (Fig. 1B). These results indicate that naringenin-7-O-glucoside significantly protected H9C2 cells from doxorubicin-induced cytotoxicity.

Then, we explored whether naringenin-7-O-glucoside has a protective effect against cardiomyocyte apoptosis. Doxorubicin-treated cells stained with the fluorescent DNA-binding dye, Hoechst 33258, displayed typical morphological features of apoptosis with sickle-shaped nuclei. Naringenin-7-O-glucoside pretreatment prevented these morphological changes (Fig. 2). Apoptosis was also analyzed by DNA ladder assay and flow

cytometry. The results showed that naringenin-7-O-glucoside treatment significantly prevented DNA fragmentation and inhibited cardiomyocyte apoptosis induced by doxorubicin (Figs. 3 and 4).

To further investigate the mechanisms of naringenin-7-O-glucoside in preventing doxorubicin-induced apoptosis in H9C2 cells, we investigated apoptosis-related gene expression. Naringenin-7-O-glucoside decreased the mRNA expression of caspase-3 and caspase-9, and increased the expression of Bcl-2 mRNA and the Bcl-2 protein level. Furthermore, we found that naringenin-7-O-glucoside upregulated the expression of HO-1, which is the microsomal inducible enzyme converting heme into carbon monoxide, free ferrous iron, and biliverdin, which is subsequently reduced to bilirubin (Maines, 2005; Ryter et al., 2006). Recent study has shown the critical importance of HO-1 expression in mediating antiapoptotic effects (Kim et al., 2006b). HO-1 overexpression could decrease apoptotic cell death by enhancing Bcl-2 and depressing caspase-3 expression (Wang et al., 2004). Induction of HO-1 by chemical inducers or selective overexpression is cytoprotective both in vitro and in vivo (Nakahira et al., 2003; Nath, 2006). In addition, it has been demonstrated that carbon monoxide might possess antiapoptotic properties (Brouard et al., 2000; Liu et al., 2003). Therefore, the specific activation of HO-1 gene expression by pharmacological modulation may represent a novel target for therapeutic intervention of cytotoxicity induced by doxorubicin. Our experiments showed that after pretreatment with naringenin-7-O-glucoside, levels of HO-1 mRNA and protein were found to be greatly elevated in cardiomyocytes. The induction of HO-1 may provide a protective effect against doxorubicin toxicity. Naringenin-7-O-glucoside significantly attenuated doxorubicin-mediated apoptotic death of
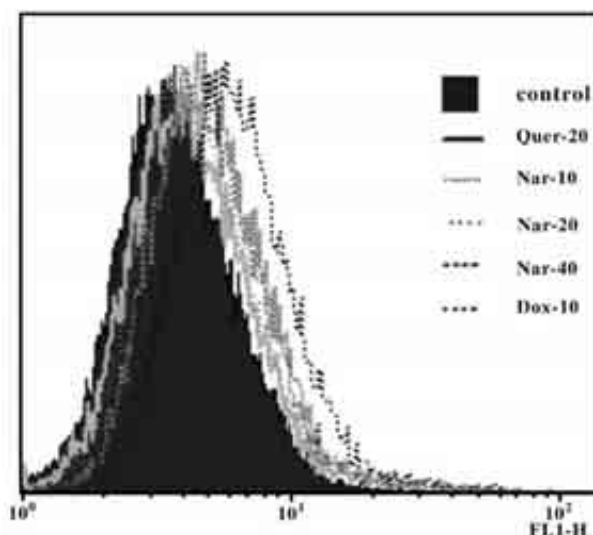

Fig. 4. Flow cytometric detection of apoptosis: FITC-Annexin V/PI double staining. Flow cytometric analysis of DNA fragmentation for H9C2 cells after naringenin-7-O-glucoside or quercetin treatment. Cells were exposed to different concentrations (10, 20, 40 μM) of naringenin-7-O-glucoside and quercetin (20 μM) for 24 h, followed by incubation with doxorubicin (10 μM) for another 24 h. After this incubation, the cells were harvested and stained with FITC-Annexin V/PI, followed by flow cytometric analysis.
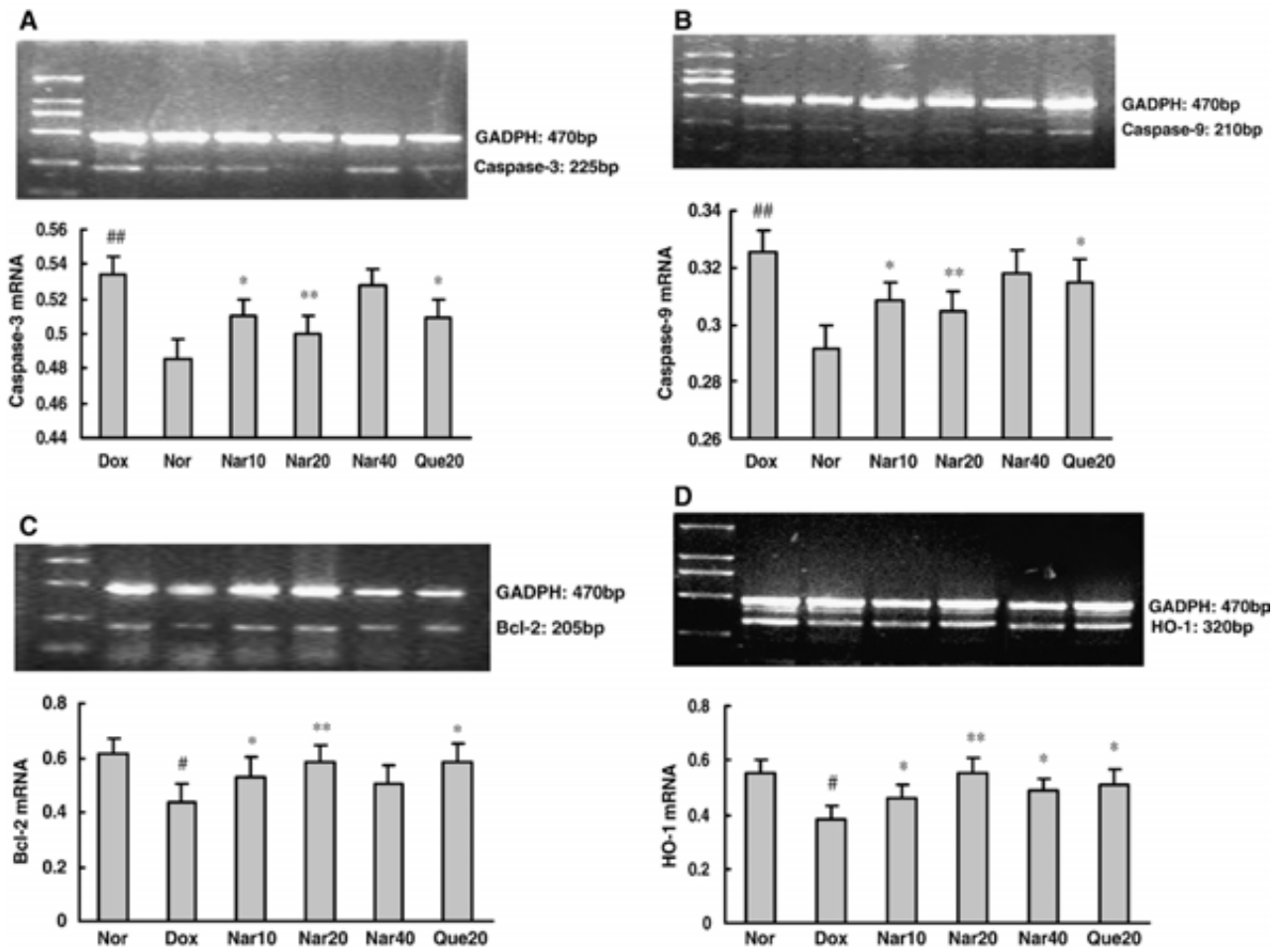
Fig. 5. Effects of naringenin-7-O-glucoside on caspase-3, caspase-9, Bcl-2 and HO-1 mRNA expression in H9C2 cells. After 24 h of treatment without (normal) or with naringenin-7-O-glucoside (10, 20, 40 μM) or quercetin (20 μM), cardiomyocytes were incubated with 10 μM doxorubicin for 24 h. Normalization relative to GAPDH was performed. Nor: normal cells; Dox: doxorubicin; Nar: naringenin-7-O-glucoside; Mar: the molecular weight markers; Que: quercetin. Results presented in bar graphs are the means±S.E.M. of four separate experiments. $^#P<0.05$, $^{##}P<0.01$ compared with normal and $*P<0.05$, $**P<0.01$ compared with doxorubicin.

H9C2 cells in a dose-dependent manner at low doses, but the effect decreased at higher doses. Taken together, it is possible to speculate that HO-1 may be actively involved in the cellular defense against the cardiotoxicity of doxorubicin, and that the induction of HO-1 might contribute to the antiapoptotic properties.
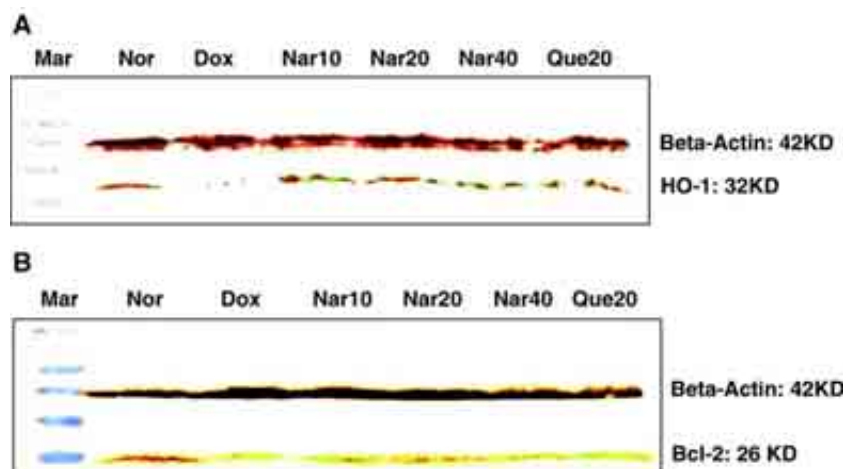

Fig. 6. Effects of naringenin-7-O-glucoside on Bcl-2 and HO-1 protein expression in H9C2 cells. After 24 h of treatment without (normal) or with naringenin-7-O-glucoside (10, 20, 40 μM) or quercetin (20 μM), cardiomyocytes were incubated with 10 μM doxorubicin for 24 h. Cell lysates were probed with antibodies against Bcl-2 and HO-1. Immunoblots shown are representative of four experiments.

In conclusion, for the first time we have found that naringenin-7-O-glucoside could ameliorate doxorubicin-induced apoptosis in H9C2 cells. The protective effects of naringenin-7-O-glucoside were not only related to modulation of apoptosis-related gene (Bcl-2, caspase-3, and caspase-9) expression, but also to modulation of HO-1 expression. These results indicate that naringenin-7-O-glucoside from *D. rupestre* Hance might be useful for treating or preventing cardiomyopathy associated with doxorubicin as a result of its antiapoptotic properties.

Acknowledgment

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