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Protective effect of bioactive compounds from *Lonicera japonica* Thunb. against H_2O_2-induced cytotoxicity using neonatal rat cardiomyocytes

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ABSTRACT

Objective(s): Pharmacological studies showed that the extracts of Jin Yin Hua and its active constituents have lipid lowering, antipyretic, hepatoprotective, cytoprotective, antimicrobial, antibiotic, antioxidative, antiviral, and anti-inflammatory effects. The purpose of the present study was to investigate the protective effects of caffeoylquinic acids (CQAs) from Jin Yin Hua against hydrogen peroxide (H_2O_2)-induced and hypoxia-induced cytotoxicity using neonatal rat cardiomyocytes.

Materials and Methods: Seven CQAs (C1 to C7) isolated and identified from Jin Yin Hua were used to examine the effects of H_2O_2-induced and hypoxia-induced cytotoxicity. We studied C4 and C6 as preventative bioactive compounds of the reactive oxygen species (ROS) production, apoptotic pathway, and apoptosis-related gene expression.

Results: C4 and C6 were screened as bioactive compounds to exert a cytoprotective effect against oxidative injury. Pretreatment with C4 and C6, dose-dependently attenuated hypoxia-induced ROS production and reduced the ratio of GSSG/GStotal. Western blot data revealed that the inhibitory effect of C4 on H_2O_2-induced up and down-regulation of Bcl-2, Bax, caspase-3, and cleaved caspase-3. Apoptosis was evaluated by detection of DNA fragmentation using TUNEL assay, and quantified with Annexin V/PI staining.

Conclusion: *In vitro* experiments revealed that both C4 and C6 protect cardiomyocytes from necrosis and apoptosis during H2O2-induced injury, via inhibiting the generation of ROS and activation of caspase-3 apoptotic pathway. These results demonstrated that CQAs might be a class of compounds which possess potent myocardial protective activity against the ischemic heart diseases related to oxidative stress.

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Introduction

The incidence of cardiovascular diseases is rising year by year; cardiovascular diseases, especially the ischemic heart diseases (IHD) have become the main cause of death worldwide (1). Based on previous studies, increased generation of reactive oxygen species (ROS) is linked to the onset and progression of IHD. Although the precise mechanism is poorly interpretive, oxidative stress appears to be involved, and plays a pivotal role in the pathophysiology of ischemic cardiac disorders. When oxygen supply to myocardial cells is deprived, the massive generation of free radicals and ROS are induced, which can attack proteins, deoxynucleic acids, and lipid membranes, thereby disrupting cellular function and and even causing the apoptosis of myocardial cells (2, 3). Moreover, inhibiting the generation of ROS has been indicated as a treatment for IHD (4).

Much more attention has been recently paid to the traditional Chinese herbal medicine (TCM); some extracts and compounds, such as Radix Salviae miltiorrhizae, have been proven to treat IHD by scavenging the endogenous ROS (5-7). However, because of the multicomponent and complicated preparing process, it was difficult to explore the pharmacological mechanism of TCM in detail. With more and more chemical compounds isolated and identified from TCM, some of them are demonstrated to be the pharmacodynamic material basis of anti-oxidant effect.

Lonicera japonica Thunb. (Jin Yin Hua), a

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traditional medicine for thousands of years in China, which has also been listed in the Pharmacopoeia of the People's Republic of China and more than 500 prescriptions containing *Jin Yin Hua* has been extensively used for acute rheumatoid arthritis, hepatitis, upper respiratory tract infections, dysentery, fever, throat inflammations, measles, chickenpox, infected wounds, and gastroenteritis. Numerous compounds, such as phenolic acids, flavonoids, alkaloids, cerebrosides, iridoids, and triterpenoid saponins have been isolated and identified from various parts of this plant (8-12). Pharmacological studies show that the extracts of *Jin Yin Hua* and its active principles have lipid lowering, antipyretic, hepatoprotective, cytoprotective, antimicrobial, antibiotic, antioxidative, antiviral, and anti-inflammatory effects (11, 13-16). Moreover, it has been also used to produce healthy beverage and tea in China.

In this study, caffeoylquinic acids (CQAs), a class of main polyhydroxy chemical compositions of the *Jin Yin Hua*, were screened against the damage mediated by oxidative stress using primary rat cardiomyocytes. We also examined the underlying anti-apoptotic mechanism, which might be related to manipulating the apoptosis-related genes such as Bcl-2, Bax, caspase-3, and cleaved caspase-3.

Materials and Methods

Extraction and isolation of compounds

The dried flower buds of *L. japonica* (3.2 kg) were refluxed with H_2O (32 l), twice for 3 hr each time. The extract was concentrated in vacuo to a volume of 2 l, and precipitated with 95% EtOH (v/v) (6 l). The supernatant was filtered and the solvent was evaporated in vacuum. Then the residue (1 kg) was subjected to column chromatography on D101 macroporous adsorption resin and eluted with EtOH/H_2O gradient. The 30% EtOH (v/v) eluate (220 g) was suspended in H_2O and partitioned with n-BuOH. The n-BuOH-soluble fraction (100 g) was chromatographed over silica gel by gradient elution with $CHCl_3$/MeOH (95:5→0:100) to give 10 subfractions. Subfraction 5 (34.5 g) was passed through a Sephadex LH-20 column with MeOH as eluent and then subjected to ODS HPLC, eluted with MeOH/H_2O (1:9→10:0). The eluate of 20% MeOH was purified by preparative HPLC eluted with 23% MeOH (1‰ HAc) to afford compound 1 (C_1) (512.5 mg). The eluate of 30% MeOH was purified by preparative HPLC, and eluted with 38% MeOH (1‰ HAc) to yield C_2, C_3, C_4, and C_5 (60.1, 42.3, 375.5, and 245.2 mg, respectively). C_6 (101.0 mg) and C_7 (171.3 mg) were finally obtained from the eluate of 40% MeOH by purification with preparative HPLC.

Compounds dilution

Each compound was first dissolved in dimethyl sulfoxide (DMSO) 0.1%, and then serially diluted in PBS to immediately prior experiments. Stock solutions of samples were used within 1 week after preparation.

Isolation and culture of neonatal rat cardiomyocytes

The cardiomyocytes were harvested from Wistar rats, 1-3 days after birth. Their ventricles were quickly removed and cut into pieces of 1 to 2 mm^3, then digested with 0.25% trypsin solution (Beyotime Institute of Biotechnology). Isolated cells from each digestion were pooled in DMEM (Hyclone Laboratories), supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 U/ml). Cardiac tissues were trypsinized until the tissues disappeared and cell suspensions were collected. Then the cell suspensions were centrifuged at 2500 rpm for 3 min, the isolated cells were seeded in a culture flask and incubated at 37 °C, in a 5% CO_2 incubator. After 90 min for fibroblast adherence, cardiomyocytes were isolated with the technique of differential anchoring velocity. Primary cardiomyocytes were incubated at 37 °C in a 5% CO_2 incubator for 48 hr, then the spent medium was removed and replaced.

Injuries of oxidative stress

Cardiomyocytes were plated in 96-well plates at a density of 1×10^4 per well for 48 hr. Cells were pretreated with different concentrations of compounds for additional 12 hr, followed by serum-free DMEM and 200 μM H_2O_2, and incubated for 12 hr. In the hypoxic environment, after plated in the 96-well plates, the cardiomyocytes were cultured in low-glucose DMEM with different concentrations of compounds (from the extract), and immediately incubated in the hypoxic chamber (5% CO_2, 3% O_2, and 92% nitrogen) for 72 hr.

Cell viability assay

Cell viability was determined by MTT assay. After H_2O_2 or hypoxia treatment, the media of the samples were changed with DMEM, and MTT was added to the cell cultures at a final concentration of 0.5 mg/ml; the mixture was incubated for 4 hr at 37 °C. The supernatant was removed and the formed formazan crystals in viable cells were solubilized in 200 μl DMSO. The value was determined by measuring the absorbance at a wavelength of 490 nm using a microplate reader. The viability of the cardiomyocytes (%) was calculated according to the following formulae: viability (% of control) = OD mean test group / OD mean control group × 100%.

TUNEL staining

Cardiomyocytes were cultured in the coverslips for 48 hr, then the medium was changed with serum-free media. C_4 and C_6 were selected and added to the medium for additional 12 hr at the final concentrations of 10 μM and 50 μM, respectively. After inducing apoptosis in cardiomyocytes with H_2O_2 for additional 4 hr, the cells were washed twice with PBS, fixed in 4% paraformaldehyde for 60 min, and permeabilized (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Finally, 50 μl of TUNEL reaction mixture was added and incubated for 60 min at 37 ˚C. The coverslips were observed under fluorescence microscope.

Assessment of apoptosis by Annexin V staining

The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (Beyotime Institute of Biotechnology) was used to detect apoptosis according to the manufacturer's instructions. The cardiomyocytes were incubated with C_4 and C_6 at final concentrations of 50 μM. After H_2O_2-induced injury, the cells were digested with trypsin and washed twice with cold PBS. The cells were resuspended in 500 μl binding buffer. 5 μl of Annexin V and 5 μl of PI were added to the cells and incubated for 15 min.

Intracellular ROS production assay

The generation of intracellular ROS was determined using the fluorescent probe DCFH-DA (Beyotime Institute of Biotechnology). Briefly, cardiomyocytes were cultured in low-glucose DMEM with C_4 and C_6 for 12 hr, then immediately cultured in the hypoxic chamber (5% CO_2, 3% O_2, and 92% nitrogen) to maintain hypoxia for 48 hr, and finally incubated with DCFH-DA in serum-free DMEM for 20 min at 37 °C. In order to remove DCFH-DA, the cells were washed three times with serum-free DMEM. The level of cellular fluorescence was measured with a fluorescence microscope. Data was calculated by subtracting the intensity of control cells, and expressed as a percentage of control.

Intracellular total glutathione (GStotal) and GSSG production assay

The GStotal and GSSG were determined using total glutathione assay kit (Beyotime Institute of Biotechnology). The cardiomyocytes were pretreated with C_4 and C_6 for 12 hr, and then subjected to oxidative damage induced by H_2O_2. All procedures completely complied with the manufacturer's instructions.

Western blot analysis

For Western blot analysis, cultured cardiomyocytes were harvested and lysed for 20 min at 4 °C in lysis buffer. Protein samples (100 g) extracted from cardiomyocytes were separated by 12% SDS-PAGE, transferred to nitrocellulose membrane, and blocked with 5% non-fat dry milk in TBST for 2 hr. After blocking, the membranes were probed with the primary antibodies. The rat monoclonal caspase-3, cleaved caspase-3, Bcl-2, and Bax primary antibodies (Cell Signaling Technology) were added to the membrane at 1:1000 dilutions in TBST and incubated at 4 ˚C overnight. After washing the membrane, the blots were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (Santa Cruz Biotechnology). Immunoreactive bands were quantified using Odyssey v1.2 software by measuring the band intensity for each group and normalizing to β-actin as an internal control.

Quantitative real-time PCR

Cardiomyocytes that had been pretreated with C_4 (10 μM and 50 μM) were subjected to 200 μM H_2O_2 for 4 hr. Cells were washed once in PBS. Total RNA was extracted using TRIZOL reagent. First-strand cDNA was reversed transcribed using oligo-dT. To detect the level of caspase-3, caspase-9, and GAPDH mRNAs, quantitative real-time PCR was performed on ABI 7500 fast Real Time PCR system (Applied Biosystems, USA). The real-time PCR primer sequences for caspase-3 were forward: 5′-TTGGAACGAACGGACCTG-3′ and reverse: 5′-TTGGAACGAACGGACCTG-3′. The real-time PCR primer sequences for caspase-9 were forward: 5′-GGCCTTCACTTCCTCTCAA-3′ and reverse primer: 5′-GCTCTTCTTGTCTCTCCAGG-3′. The primer sequences for GAPDH were forward primer: 5′-TCTACATGTTCCAGTATGACTC-3′ and reverse primer: 5′-ACTCCACGACATACTCAGCACC-3′. GAPDH was used as an internal control.

Statistical analysis

Data were statistically analyzed using one-way ANOVA and expressed as the mean±SEM. All statistical analyses were performed by SPSS version 17 (P<0.05).

Results

Structural identification of caffeoylquinic acids (C_1-C_7)

The chemical structures of CQAs **(C_1 to C_7)** are exhibited in Figure 1A. C_1 was obtained as a white amorphous powder. The ESIMS (*m/z* 355.1 $[M+H]^+$, *m/z* 377.1 $[M+Na]^+$, 352.9 $(17)^-$) and NMR analyses revealed the molecular formula as $C_{16}H_{18}O_9$. The ^1H NMR and ^{13}C NMR spectra of C_1 displayed the presence of a caffeoyl moiety and a quinic acid moiety. By comparison of data with the literature, C_1 was identified as chlorogenic acid (18, 19).

$C_{17}H_{20}O_9$ by ESIMS (*m/z* 369.1 $[M+H]^+$, *m/z* 391.1 C2 was obtained as a yellowish amorphous powder. Its molecular formula was

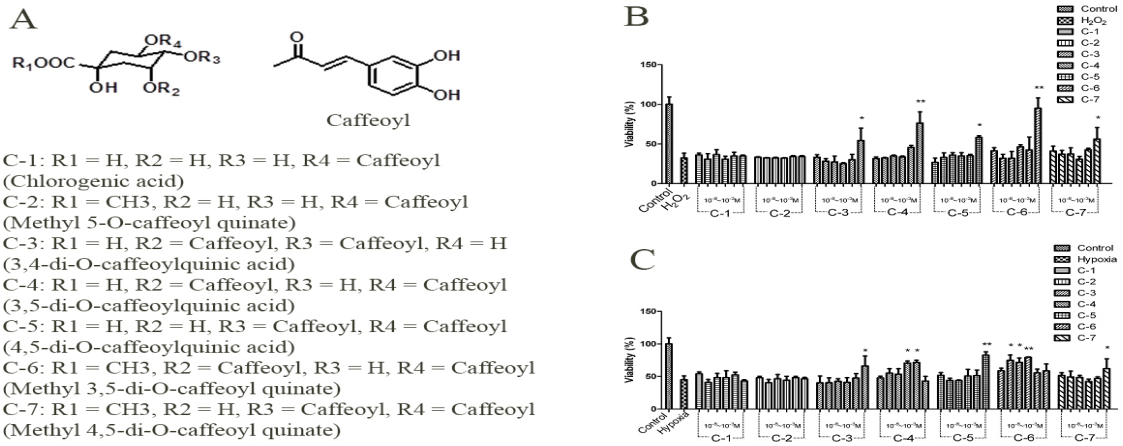

Figure 1. Protective effect against H_2O_2-induced and hypoxia-induced cytotoxicity in caffeoylquinic acids-treated primary cultured cardiomyocytes. A, structural identification of CQAs (1-7). B, the cells viability was measured following H_2O_2-induced myocardial injury. CQAs increased the cell viability, n=3. C, the cells viability was measured following hypoxia-induced myocardial injury. CQAs increased the cell viability, (n=3; *, P<0.05). Data are means±SEM of tree independent experiments

determined as $[M+Na]^+$, *m/z* 367.0 $(17)^-$) and NMR analyses. The ^1H NMR and ^{13}C NMR spectra of C_2 were similar to those of C_1, except for an additional methoxyl signal. The carbon signal for the carboxyl group of the quinic acid moiety in C_2 was shifted upfield relative to that in C_1, suggesting that C_2 was the methyl ester of C_1. In accordance with the literature data, the structure of C_2 was determined as methyl 5-*O*-caffeoyl quinate (18).

C_3 was obtained as a yellowish amorphous powder, with the molecular formula of $C_{25}H_{24}O_{12}$ established by ESIMS (*m/z* 515.0 $(17)^-$) and NMR analyses. The ^1H NMR and ^{13}C NMR spectra of C_3, showed the presence of two caffeoyl moieties and a quinic acid moiety, deducing that C_3 was a di-*O*-caffeoylquinic acid. The locations of two caffeoyl moieties on the quinic acid moiety were deduced from the downfield shifts of the protons at H_3 and H_4 in C_3 as compared to those in the free quinic acid. Therefore, C_3 was identified as 3,4-di-*O*-caffeoylquinic acid. The ^1H NMR and ^{13}C NMR data of C_3 were in good agreement with those reported in the literature (19).

C_4 and C_5 were obtained as white amorphous powders, with the same molecular formula of $C_{25}H_{24}O_{12}$ as C-3. The ^1H NMR and ^{13}C NMR spectra of C_4 and C_5 were similar to those of C_3, which exhibited typical signals for di-*O*-caffeoylquinic acids. The positions of two caffeoyl moieties on the quinic acid moiety were determined by comparison of the chemical shifts of the protons of the quinic acid moieties between C_4 and C_5 and the free quinic acid respectively. Thus, C_4 and C_5 were established as 3,5-di-*O*-caffeoylquinic acid and 4,5-di-*O*-caffeoylquinic acid, respectively. The ^1H NMR and ^{13}C NMR data of C_4 and C_5 were in good agreement with those reported in the literature (20).

C_6 was obtained as a yellowish amorphous powder. The molecular formula of $C_{26}H_{26}O_{12}$ was determined by ESIMS (*m/z* 529.0 (17)) and NMR analyses. The ^1H NMR and ^{13}C NMR spectra of C_6 were similar to those of C_4, except for an additional methoxyl signal. The carbon signal for the carboxyl group of the quinic acid moiety in C_6 was shifted upfield as compared to that in C_4, suggesting that C_6 was the methyl ester of C_4. Thus, C_6 was determined as methyl 3,5-di-*O*-caffeoyl quinate (21).

C_7, yielded as a white amorphous powder, had a molecular formula of $C_{26}H_{26}O_{12}$ by analyses of ESIMS (*m/z* 529.0 (17)) spectrum and NMR. The ^1H NMR and ^{13}C NMR spectra of C_7 were similar to those of C_5, except for an additional methoxyl signal. The carbon signal for the carboxyl group of the quinic acid moiety in C_7 was shifted upfield as compared to that in C_5, suggesting that C_7 was the methyl ester of C_5. In accordance with the literature data, the structure of C_7 was identified as methyl 4,5-di-*O*-caffeoyl quinate (21).

Effect of caffeoylquinic acids on H_2O_2 and hypoxia-induced apoptosis

As shown in Figure 1B, cardiomyocytes viability fell to 32.2±6.1% following exposure to 200 μM H_2O_2 for 12 hr. Treatment with different concentrations of compounds attenuated H_2O_2 cytotoxicity. Compounds of C_3 to C_7 at a concentration of 1×10^{-3} M significantly increased cell viability to 54.3±15.7%, 76.4±14.1%, 58.2±2.1%, 95.0±13.1%, and 56.1±14.4%, respectively. As shown in Figure 1C, incubation in hypoxic chamber (5% CO_2, 3% O_2, and 92% nitrogen) for 72 hr, decreased the viability of cardiomyocytes cells to 45.2±5.8%. Treatment with different concentrations of compounds attenuated hypoxia cytotoxicity. C_3, C_5, and C_7 at a concentration

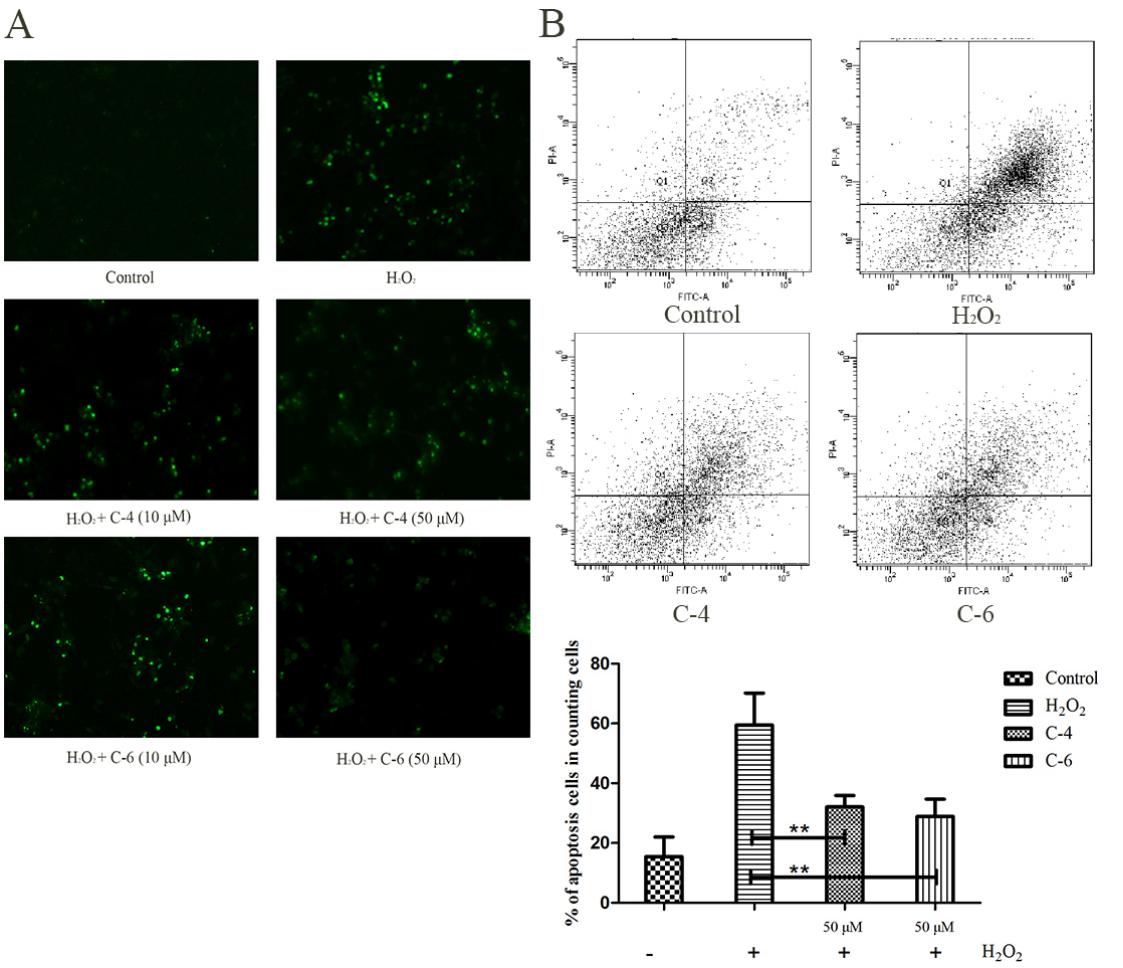

Figure 2. Anti-apoptotic effects of C4 and C6 in cardiomyocytes following H_2O_2 stimulation. A, TUNEL-positive cardiomyocytes were significantly more than that treated with C4 and C6. B, quantitative analysis of apoptotic cardiomyocytes by flowcytometry. Treatment of cardiomyocytes with C4 and C6 resulted in anti-apoptotic effect, (n=3; **, P<0.01). Data are mean±SEM

of 1×10^{-3} M significantly increased viability to 62.3±15.3%, 77.8±5.2%, and 58.5±15.0%, respectively. C_4 increased cell viability at concentrations of 1×10^{-5} M and 1×10^{-4} M to 75.8±3.4% and 76.6±3.6%, respectively. In addition, C_6 at concentrations of 1×10^{-7} M, 1×10^{-6} M, and 1×10^{-5} M also increased cell viability to 70.5±8.2%, 67.4±6.8%, and 74.7±0.8%, respectively.

C_4 and C_6 protect cardiomyocytes from H_2O_2-induced apoptosis

Based on the results of cardiomyocytes viability, C_4 and C_6 had protective effect against H_2O_2-induced and hypoxia-induced damages at a lower concentration. In this case, C_4 and C_6 were used to observe whether CQAs could decrease H_2O_2-induced cardiomyocyte apoptosis with the in situ assay. As shown in Figure 2A, cardiomyocytes were subjected to treatment with CQAs for 12 hr, followed by H_2O_2 for 4 hr; the TUNEL-positive cardiomyocytes were significantly increased compared with control group. In contrast, treatment with C_4 and C_6 concentration-dependently reduced the number of TUNEL-positive cardiomyocytes. For quantitative analysis of anti-apoptotic effects, flowcytometry analysis of Annexin V/PI staining was utilized. As shown in Figure 2B, 59.5% of cells were in the apoptotic stage after exposure to H_2O_2 for 4 hr. C_4 reduced the percentage of apoptotic cells to 32.1% and C_6 to 28.9%.

Effects of C_4 and C_6 on the production of intracellular ROS, Gstotal, and GSSG

The generation of ROS during oxidative metabolism within the cardiomyocytes are an important biomarker of oxidative stress. The GSH serves as an electron donor against the radical formation, and changes into GSSG. As shown in Figure 3A, the intracellular ROS level is indicated by the fluorescent probe DCFH-DA. Pretreatment with C_4 and C_6, dose-dependently attenuated hypoxia-induced ROS production. As shown in Figure 3B, the ratio of H_2O_2-induced GSSG/GStotal in cardiomyocytes

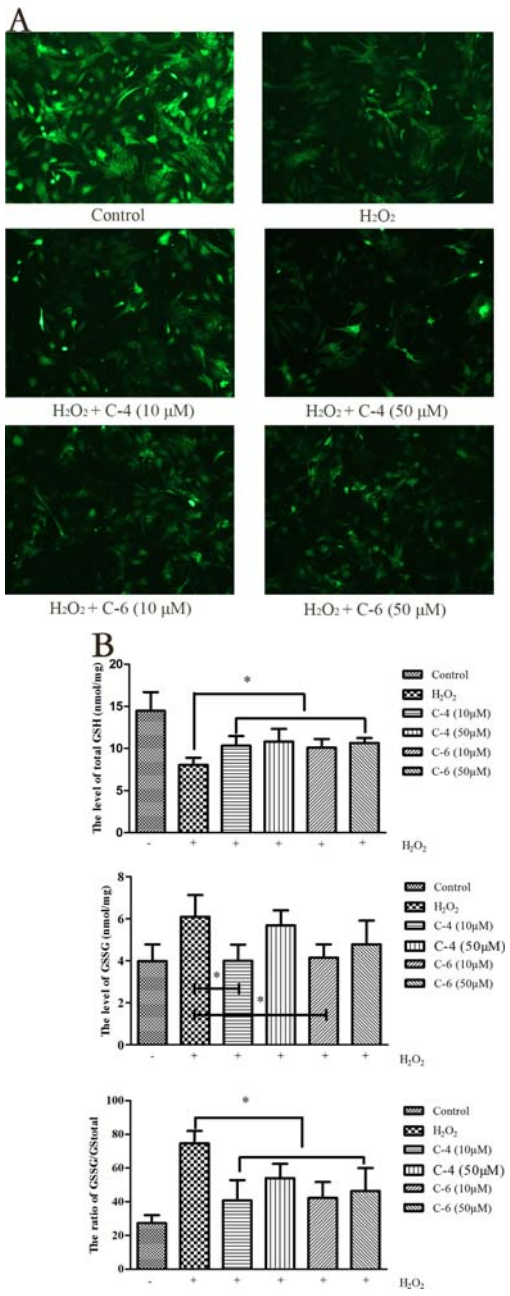

Figure 3. C4 and C6 enhanced the cellular adaptation to hypoxia. A, the fluorescent probe DCFH-DA indicated the intracellular ROS level; the green fluorescence intensity was proportional to the production of ROS. Treatment of cardiomyocytes with C4 and C6 decreased the production of hypoxia-induced intracellular ROS. B, bar chart shows the GS total and GSSG levels; C4 and C6 inhibited the production of hypoxia-induced intracellular ROS by increasing the GS total and decreasing the ratio of GSSG/ GStotal (n=3; *, P<0.05). Data are mean±SEM

increased to 74.6±12.8%. Pretreated with 10 μM and 50 μM of C_4 and C_6, C_4 reduced the ratio of GSSG/GStotal to 40.8±20.9%, 54.1±14.8%, and C_6 reduced the ratio of GSSG/GStotal to 42.3±16.3%, and 46.4±23.6% respectively.

Effects of C_4 on expression of apoptotic proteins

As shown in Figure 4A, H_2O_2 significantly increased Bax and decreased Bcl-2 expressions. Pretreatment with C_4, 50 μM for 12 hr, increased Bcl-2 and decreased Bax expressions. These results suggested that C_4 inhibited ROS induced apoptosis by decreasing the ratio of Bax/Bcl-2. We investigated the level of the active p19 subunit of caspase-3 (cleaved caspase- 3), the cleaved caspase-3 indicated the proteolytic activation of caspase-3. As shown in Figure 5A, compare to control group, an obvious decrease of caspase-3 expression but an increase of cleaved caspase-3 were detected after injury induced by H_2O_2. However, C_4 revealed enhanced caspase-3 activity and reduced cleaved caspase-3 expression compared with the H_2O_2 groups, in a concentration-dependent manner. These results suggested that C_4 decreased cleaved caspase-3 expression to inhibit oxidative stress-induced apoptosis.

Effect of C_4 on caspase-3 and caspase-9 mRNA expression in the cardiomyocytes

The expression levels of key apoptotic-related genes caspase-3 and caspase-9 were assessed. Our results indicated that H_2O_2 treatment was associated with significant decreases in the expression of caspase-3 and caspase-9. Compared with the H_2O_2 group, the expression of caspase-3 and caspase-9 in the C_4 group was up-regulated in a dose-independent manner. As shown in Figure 4B.

Discussion

In the present study, seven CQA derivatives (C_1-C_7) were isolated from the aqueous extract of the flower buds of *L. japonica*. 3,5-di-*O*-caffeoylquinic acid (C_4) and methyl 3,5-di-*O*-caffeoyl quinate (C_6) were screened as the active compounds against H_2O_2-induced and hypoxia-induced oxidative injuries, by MTT assay. Furthermore, *in vitro* experiments revealed that both C_4 and C_6 protected cardiomyocyte from necrosis and apoptosis during H_2O_2-induced injury via inhibiting the generation of ROS and activation of caspase-3 apoptotic pathway.

In recent years, considerable attention has focused on the material basis of traditional Chinese medicine. The material basis of *Jin Yin Hua* responsible for its antioxidative effect has been be attributed to phenolics including chlorogenic acid (22). Researches of such compounds are mainly concentrated on the caffeoylquinic acid and dicaffeoylquinic. Phytochemical studies proposed *Jin*

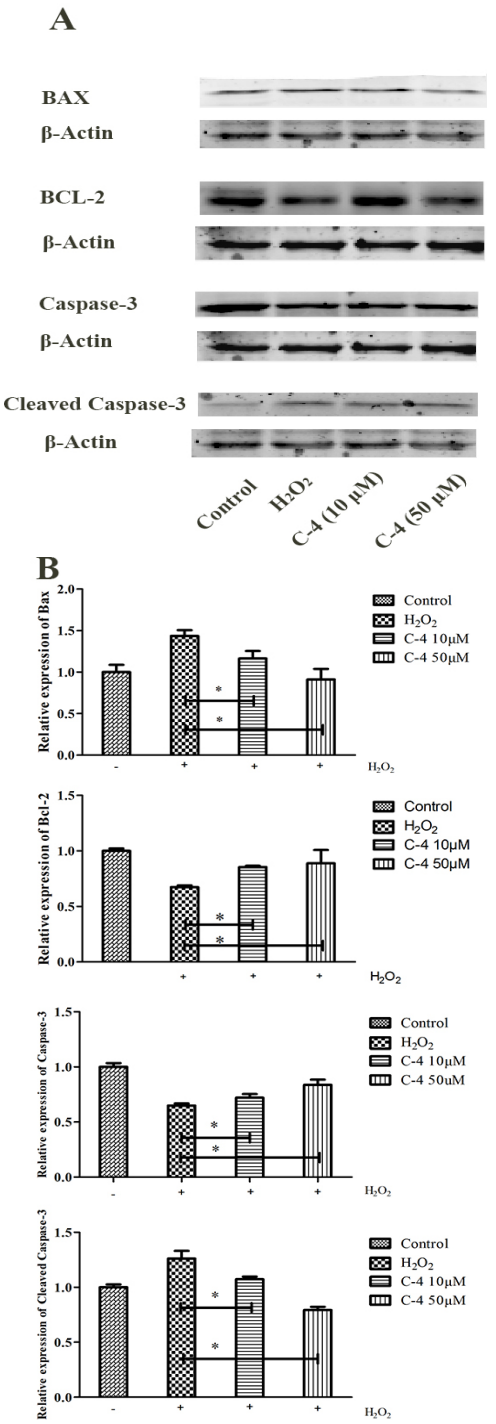

Figure 4. Inactivation of caspase-3 cell apoptosis pathway is involved in C4 treated cardiomyocytes following H2O2 stimulation. A, Western blot analysis of the expression and phosphorylation levels of caspase-3 cell apoptosis pathway. B, bar chart shows the effect of C4 on expression of apoptotic proteins (n=3; *, *P*<0.05). Data are mean±SEM

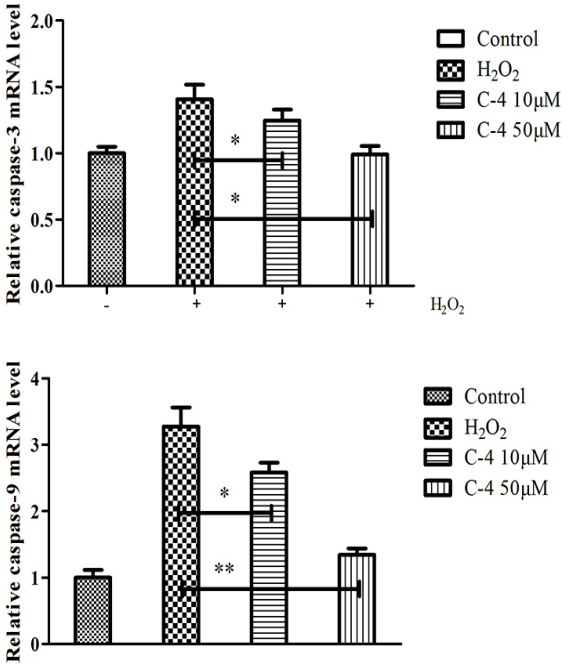

Figure 5. Quantitative real-time PCR of apoptotic genes, caspase-3 and caspase-9, in C4 treated cardiomyocytes following H_2O_2 stimulation (n=3; *, P <0.05). Data are mean ± SEM

Yin Hua a convenient source of CQA, including chlorogenic acid, 1-*O*-CQA, 4-*O*-CQA, 4,5-di-*O*-CQA, 3,5-di-*O*-CQA, 1,3-di-*O*-CQA, 3,4-di-*O*-CQA, 1,4-di-*O*-CQA and 3,4-di-*O*-CQA methyl ester, etc (23, 24). Although CQA and their derivatives have diverse bioactivities, their antioxidative effects have drawn much attention (25, 26).

It is widely accepted that oxidative stress, which is associated with increased formation of ROS, plays a major role in a variety of cardiovascular diseases, and is an important factor leading to cardiomyocyte apoptosis (27, 28). Although there is no such study on the protective effect of CQA derivatives on ROS-induced cardiomyocytes apoptosis, these compounds have exhibited remarkable anti-apoptotic properties in several other cell lines because of their antioxidative activity (25). *In vitro*, we established H_2O_2-induced and hypoxia-induced apoptosis in primary neonatal rat cardiomyocytes to evaluate the protective effect of CQAs. As we know, H_2O_2 causes lipid peroxidation and DNA damage, which induce apoptosis in neonatal rat cardiomyocytes. Hypoxia of cardiomyocytes can mimic the condition of IHD *in vitro*, and induce endogenous ROS production and accumulation in the cells via intrinsic mitochondrial pathway (29). It has been indicated that some CQAs can effectively protect cardiomyocytes against oxidative stress in a dose-dependent manner. As typical representatives of CQAs, C_4 and C_6 significantly decreased H_2O_2-induced GSSG production, which defends cells against H_2O_2-induced damages. GSH, the reduced glutathione, serves as a central component of the cellular

antioxidant defense, and detoxifies ROS to generate the GSSG, as a central component of the cellular antioxidant defense (30).

To explore the anti-apoptotic effect of C_4 and C_6, the TUNEL and flowcytometry analyses were performed. The results demonstrated that these two CQA derivatives could reduce the H_2O_2-induced cardiomyocyte apoptosis, in a dose-dependent manner. The cleavage of caspase-3 is considered to be an important process in activating the apoptotic signaling pathway, and a potential therapeutic target for preventing cardiomyocyte apoptosis (31). The Bcl-2 protein family also plays a central role in the transition of apoptotic signals towards the mitochondria during oxidative stress-induced apoptosis (32). By investigating the expression of the apoptotic proteins caspase-3, cleaved caspase-3, and the ratio of Bax/Bcl-2, we found that the cleaved caspase-3 and the ratio of Bax/Bcl-2 were significantly up-regulated by H_2O_2-induced cardiomyocyte injury, while significantly down-regulated by treatments with C_4. Our *in vitro* experiments showed that the cardioprotection of CQAs contributed to reducing the oxidative stress-induced cell damage, which might inhibit the activation of the apoptotic pathway.

Conclusion

Findings of this study demonstrated that CQAs significantly protect cultured cardiomyocytes from oxidative stress-induced injury and decrease apoptosis *in vitro*. The underlying mechanism might be associated with their antioxidative capacity. Different protective effects of CQA derivatives on cardiomyocytes could be related to constituents' structure and location. CQAs are among major active ingredients of *Jin Yin Hua*, which might be used to protect cardiomyocytes against oxidative stress-induced damages.

Conflict of interest

The author declare that they have no conflict of interest to disclose.

Aknowledegment

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