

Protective effects of carnosic acid against mitochondria-mediated injury in H9c2 cardiomyocytes induced by hypoxia/reoxygenation

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Abstract. Myocardial ischemia and reperfusion occurs in myocardial infarction. Timely reperfusion will exacerbate the injury through the mitochondria-mediated apoptosis in cardiomyocytes due to the accumulation of excessive reactive oxygen species (ROS). In order to identify novel therapeutic approaches, the cardioprotective effects of carnosic acid and the underlying mechanisms were investigated in the present study in H9c2 cardiomyocytes injured by hypoxia/reoxygenation *in vitro*. The viability of H9c2 cardiomyocytes was detected by MTT assay and further confirmed by the detection of intracellular lactate dehydrogenase (LDH) release. The mitochondrial function in H9c2 cardiomyocytes was evaluated using fluorescence methods. The proteins related to apoptosis, including caspase-3, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax) were analyzed by western blot analysis, and the activity of caspase-3 was determined using a colorimetric method. As a result, carnosic acid was demonstrated to improve the viability of H9c2 cardiomyocytes and suppress the leakage of cytosolic lactate dehydrogenase under hypoxia/reoxygenation. In addition, the overproduction of intracellular ROS and intracellular calcium overload were ameliorated in the presence of carnosic acid. The dysfunction of mitochondria in H9c2 cardiomyocytes was also attenuated by carnosic acid through blocking the collapse of mitochondrial membrane potential (MMP) and mitochondrial permeability transition pore (mPTP) opening. Furthermore, the apoptosis of H9c2 cardiomyocytes resulted from hypoxia/reoxygenation was inhibited by carnosic acid through the upregulation of Bcl-2 and the downregulation of Bax and caspase-3 levels. These results provided evidence for further investigation

that would assist in the development of novel therapeutic approaches for myocardial infarction.

Introduction

Ischemic heart disease is one of the major causes of mortality in humans worldwide (1). The ischemic myocardium will finally lead to myocardial infarction. However, timely reperfusion exacerbates the heart failure in patients, though the infarct size has not enlarged (2). Post-ischemic reperfusion contributes greatly to the cardiomyocyte death through the mitochondria-mediated pathway (3). The mechanisms of myocardial ischemia and reperfusion injury include overproduction of reactive oxygen species (ROS), overload of intracellular calcium, collapse of mitochondrial membrane potential (MMP) and prolonged opening of the mitochondrial permeability transition pore (mPTP) among other processes (4). Natural compounds, such as berberine (5), tanshinone IIA (6) and lycopene (7), serve a pivotal role in the development of effective therapeutics for myocardial ischemia and reperfusion injury.

Carnosic acid is a natural diterpenoid (Fig. 1) that has been identified as the major bioactive phytochemical in numerous medicinal plants, including *Rosmarinus officinalis* (8), *Salvia fruticosa* (9) and *Ocimum sanctum* (10). Previous pharmacological studies have demonstrated that carnosic acid affords various biological activities, such as neuroprotection (11), prevention of advanced glycation end-product formation (12), attenuation of Alzheimer's disease (13), anticancer activity (14), anti-inflammation (15) and renoprotection (16). Furthermore, carnosic acid presented a cardioprotective effect in an isoproterenol-induced myocardial stress mouse model via preventing oxidative stress and apoptosis (17).

In an attempt to identify novel therapeutic approaches for myocardial ischemia and reperfusion injury, the present study assessed the myocardial protection exerted by carnosic acid and the associated underlying mechanisms using H9c2 cardiomyocytes subjected to hypoxia/reoxygenation.

Materials and methods

Chemicals and reagents. Carnosic acid was purchased from J&K Scientific Ltd. (Beijing, China). Dulbecco's

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modified Eagle's medium (DMEM) and fetal bovine serum were supplied by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). The Fluo-3 acetoxymethyl (AM), ROS assay kit, lactate dehydrogenase (LDH) activity assay kit, BCA protein concentration assay kit, MMP assay kit with JC-1, and Caspase-3 Activity assay kit, as well as the cleaved caspase-3 (cat no. AF0081), B-cell lymphoma 2 (Bcl-2; cat no. AF0060), Bcl-2-associated X protein (Bax; cat no. AF0054) and β -actin (cat no. AF003) antibodies, were obtained from Beyotime Institute of Biotechnology (Nantong, China). Calcein-AM was obtained from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan).

Cell culture and model establishment. Rat H9c2 cardiomyocytes were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin under humid conditions with 5% CO₂ and 95% air at 37°C. Next, the cells in logarithmic phase were incubated in 96-well plates at a density of 1x10⁵ cells/ml. The cells were divided into the control group, hypoxia/reoxygenation model group (H/R group), and three experimental groups pretreated with 0.1, 1 and 10 μ M carnosic acid in DMSO for 4 h prior to hypoxia/reoxygenation. To establish the hypoxia/reoxygenation model, H9c2 cells in the H/R and experimental groups were incubated in an atmosphere with 95% N₂ and 5% CO₂ at 37°C for 4 h, and then exposed to 95% air and 5% CO₂ at 37°C for a further 4 h. The control group was cultured under normoxic conditions.

Cell viability assay. To detect the protective effects of carnosic acid on H9c2 cardiomyocytes exposed to hypoxia/reoxygenation, an MTT assay was performed. Subsequent to the aforementioned treatments, the cells were incubated with 0.2 ml MTT (0.5 mg/ml) for 4 h at 37°C. Next, 200 μ l DMSO was added into each well in order to dissolve the formazan crystals. The optical density (OD) was recorded on a BioTek ELx800 microplate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 490 nm. The results are expressed as the relative percentage of the control group.

Extracellular LDH activity. To further confirm the protective effects of carnosic acid, the LDH activity in the culture medium was measured by an LDH assay kit, according to the manufacturer's protocol. Briefly, following treatment, the culture medium was centrifuged at 400 x g and room temperature for 5 min, and then 20 μ l supernatant was collected and mixed with 20 μ l 2,4-dinitrophenylhydrazine. After incubation at 37°C for 15 min, 250 μ l NaOH (0.4 M) was added into the mixture and incubated for a further 15 min at 37°C. The mixture was maintained at room temperature for 5 min, and subsequently the OD was recorded on a microplate reader at 450 nm. The activity of LDH was derived from the OD values and expressed as U/l.

Intracellular calcium level. To monitor the intracellular calcium in H9c2 cardiomyocytes, the fluorescence dye

Fluo-3 AM was employed, following the manufacturer's protocol. Briefly, the pretreated H9c2 cardiomyocytes were loaded with 5 μ M Fluo-3 AM at 37°C for 30 min in the dark, and then washed with PBS for three times to remove any excessive dye. The fluorescence intensity of Fluo-3 chelated with calcium was recorded on a PerkinElmer EnVision fluorescence microplate reader (PerkinElmer, Llantrisant, UK) at excitation and emission wavelengths of 488 and 525 nm, respectively.

Measurement of ROS production. The production of ROS was detected by a fluorescence method using a ROS assay kit. Subsequent to treatment, the medium was replaced and the cells were rinsed with PBS. Next, 10 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in DMEM was added and incubated at 37°C for 30 min. The cells were then washed again with PBS to remove the excessive dye, and the fluorescence intensity was recorded on a fluorescence microplate reader at 485 nm (excitation wavelength) and 520 nm (emission wavelength).

MMP assay. The MMP was also detected using a MMP assay kit with JC-1. JC-1 accumulates in the mitochondrial matrix of normal cells and forms aggregates, which emit red fluorescence under excitation. When the MMP collapses, JC-1 exists as a monomer and thus no red fluorescence is observed under excitation (18). Following the treatment, the H9c2 cardiomyocytes were washed with PBS and then loaded with JC-1 at 37°C for 20 min. Subsequent to washing with JC-1 buffer solution, the fluorescence intensity was read at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The MMP is expressed as a percentage compared with the fluorescence intensity of the control group.

mPTP opening. The mPTP opening was directly evaluated through monitoring the release of mitochondrial calcein. Briefly, cardiomyocytes were incubated with 2 μ M calcein-AM and 1 mM CoCl₂ at room temperature for 30 min. The free calcein-AM and CoCl₂ were washed with PBS to be removed, and the cells were incubated with 1 mM CoCl₂ for a further 20 min at 37°C to specifically quench the fluorescence of free calcein in the cytosol. Subsequently, the fluorescence intensity of mitochondrial calcein in the cardiomyocytes was measured on a fluorescence microplate reader at 490 nm for excitation and 515 nm for emission. The loss of calcein fluorescence in cardiomyocytes indicated the opening of mPTP. The results are expressed as the fluorescence intensity percentage of the control group.

Caspase-3 activity. Caspase-3 activity was assessed by a colorimetric assay kit following the manufacturer's instructions. H9c2 cardiomyocytes were pretreated as described earlier and washed with PBS, followed by lysis using a Caspase-3 Assay kit (cat no. C1116; Beyotime Institute of Biotechnology) and centrifugation at 16,000 x g for 10 min at 4°C. The supernatant was then collected and incubated with substrate (Ac-DEVD-pNA) at 37°C for 2 h. The OD values were measured on a microplate reader at 405 nm, and the activity of caspase-3 is expressed as the relative percentage of the OD value of the control group.

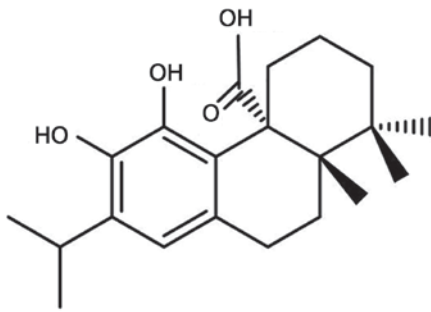


Figure 1. Chemical structure of carnosic acid.

Western blot analysis. H9c2 cardiomyocytes were treated as described earlier and then subjected to western blot analysis to detect the expression levels of caspase-3, Bcl-2 and Bax. Briefly, the cells were lysed with lysis buffer solution containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton and 1 mM phenylmethane sulfonyl fluoride on ice for 30 min. Next, the cell lysate was centrifuged at 12,000 x g for 15 min at 4°C, and the supernatant was collected as the total protein for the subsequent analysis of cleaved caspase-3, Bcl-2, and Bax. Following quantification by a BCA assay kit, the samples were separated by 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Subsequent to blocking with defatted milk at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies against cleaved caspase-3, Bcl-2, Bax and β -actin (diluted at 1:1,000). The membranes were then incubated with the respective secondary antibody conjugated to horseradish peroxidase (diluted at 1:3,000; cat no. A0192; Beyotime Institute of Biotechnology) at room temperature for 1 h, and visualized by an enzyme-link chemiluminescence substrate (cat no. P0203; Beyotime Institute of Biotechnology) on a Bio-Rad ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). β -actin was used as the internal control.

Statistical analysis. The results are expressed as the mean \pm standard deviation, and were analyzed by GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons were implemented by Student's t-test for single components or one-way analysis of variance followed by Dunnett's test for multiple components. $P < 0.05$ was considered to be an indicator of statistically significant differences. All data are the result of at least six independent experiments.

Results

Carnosic acid improves the viability of H9c2 cardiomyocytes induced by hypoxia/reoxygenation. As shown in Fig. 2, the viability of H9c2 cardiomyocytes was decreased when exposed to hypoxia/reoxygenation ($P < 0.001$). In the presence of different carnosic acid concentrations, the poor survival of H9c2 cardiomyocytes was significantly ameliorated compared with the H/R group, in a dose-dependent manner. In particular, the viability in the group treated with 10 μ M carnosic acid reached $81.55 \pm 7.31\%$ of the control group value ($P < 0.001$). These results indicated that carnosic acid exhibited

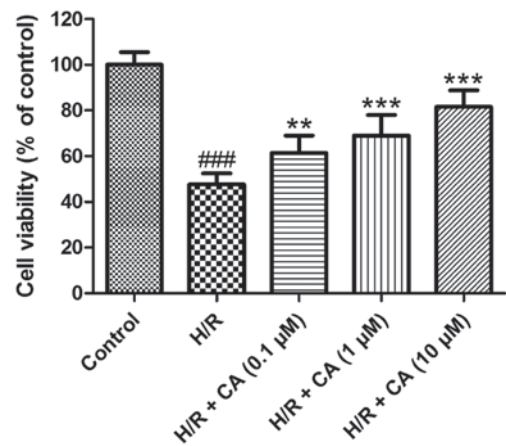


Figure 2. Effect of CA on H9c2 cardiomyocyte viability examined by an MTT assay (n=6). ### $P < 0.001$ vs. control group; ** $P < 0.01$ and *** $P < 0.001$ vs. H/R group. H/R, hypoxia/reoxygenation; CA, carnosic acid.

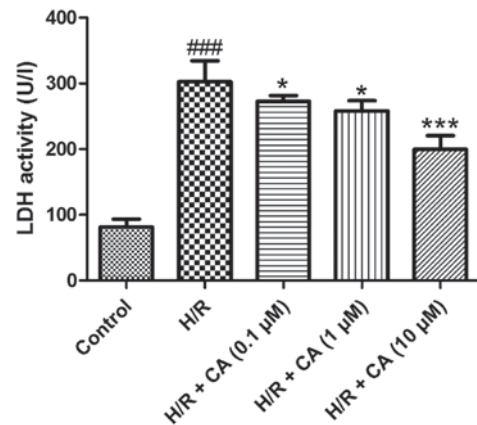


Figure 3. Effect of carnosic acid on the leakage of intracellular LDH (n=6). ### $P < 0.001$ vs. control group; * $P < 0.05$ and *** $P < 0.001$ vs. H/R group. LDH, lactate dehydrogenase; H/R, hypoxia/reoxygenation; CA, carnosic acid.

protective effects on H9c2 cardiomyocytes injured by hypoxia/reoxygenation.

Carnosic acid reduces LDH release in H9c2 cardiomyocytes induced by hypoxia/reoxygenation. Under hypoxia/reoxygenation, the activity of LDH in the culture medium of H9c2 cardiomyocytes increased to approximately three times greater than the control group activity, which demonstrated that leakage of LDH from the cytosol occurred in the H/R group and cell survival decreased ($P < 0.001$). When treated with carnosic acid, the release of LDH in H9c2 cardiomyocytes was significantly decreased in a dose-dependent manner ($P < 0.05$; Fig. 3). These results indicated the carnosic acid affected the LDH release in H9c2 cardiomyocytes induced by hypoxia/reoxygenation.

Carnosic acid attenuates the overload of intracellular calcium in H9c2 cardiomyocytes induced by hypoxia/reoxygenation. To determine the intracellular calcium level, the fluorescence probe Fluo-3 AM was used. Following treatment of hypoxia/reoxygenation, the fluorescence intensity of the intracellular calcium in the H/R group was significantly

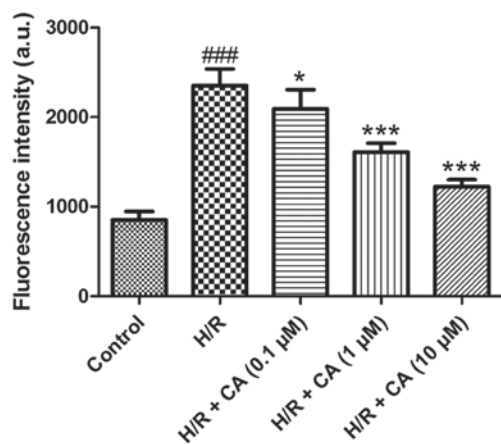


Figure 4. Effect of carnosic acid on the intracellular calcium overload in terms of the fluorescence intensity using the probe Fluo-3 acetoxymethyl (n=6). ###P<0.001 vs. control group; *P<0.05 and ***P<0.001 vs. H/R group. H/R, hypoxia/reoxygenation; CA, carnosic acid.

elevated ($2,352.85 \pm 185.48$) when compared with the control group (850.00 ± 96.86 ; $P < 0.001$; Fig. 4). However, compared with the H/R group, carnosic acid treatment dose-dependently attenuated the overload of intracellular calcium as observed by the reduced fluorescence intensity ($2,090.10 \pm 218.08$, $1,609.07 \pm 98.73$ and $1,224.07 \pm 76.41$ at 0.1, 1 and 10 μM , respectively; $P < 0.05$; Fig. 4). These results suggested that carnosic acid was able to reduce the overload of intracellular calcium in H9c2 cardiomyocytes induced by hypoxia/reoxygenation.

Carnosic acid alleviates the overproduction of ROS in H9c2 cardiomyocytes induced by hypoxia/reoxygenation. The production of intracellular ROS was measured with the fluorescence probe DCFH-DA according to the manufacturer's protocol. DCFH-DA passes through the cellular membrane and is hydrolyzed to DCFH by intracellular esterases. DCFH is then unable to cross the membrane and is thus reserved in the cytosol. With the excessive production of ROS, DCFH is quantitatively oxidized into the fluorescent dichlorofluorescein (DCF) (19). The results of the present experiments revealed that the fluorescence intensity in the H/R group (238.12 ± 20.51) was markedly higher in comparison with that in the control group (90.00 ± 9.43). Upon treatment with different concentrations of carnosic acid, the fluorescence intensity of DCF was reduced in a dose-dependent manner (Fig. 5), implying that carnosic acid was able to alleviate the overproduction of intracellular ROS in H9c2 cardiomyocytes induced by hypoxia/reoxygenation.

Carnosic acid ameliorates the collapse of MMP in H9c2 cardiomyocytes induced by hypoxia/reoxygenation. The MMP in H9c2 cardiomyocytes was evaluated to investigate the effects of carnosic acid on the mitochondrial function of H9c2 cardiomyocytes induced by hypoxia/reoxygenation. In contrast to the control group, the fluorescence intensity in the H/R group ($42.78 \pm 6.54\%$) was evidently diminished ($P < 0.001$), which implicated the collapse of MMP in the H/R group after induction of hypoxia/reoxygenation. However, in the presence of different carnosic acid concentrations, the fluorescence intensity was significantly increased to different

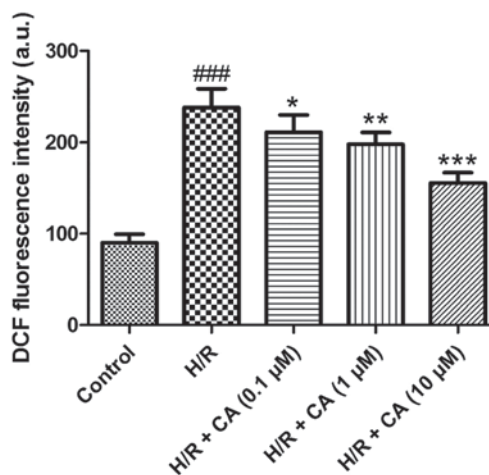


Figure 5. Effect of carnosic acid on the overproduction of intracellular ROS (n=6). ###P<0.001 vs. control group; *P<0.05, **P<0.01 and ***P<0.001 vs. H/R group. ROS, reactive oxygen species; DCF, dichlorofluorescein; H/R, hypoxia/reoxygenation; CA, carnosic acid.

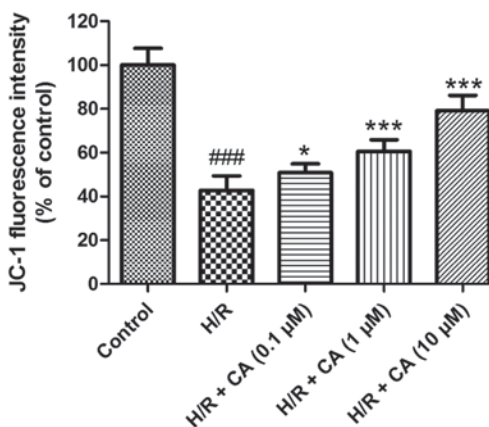


Figure 6. Effect of carnosic acid on MMP, according to the fluorescence intensity of JC-1 (n=6). ###P<0.001 vs. control group, *P<0.05 and ***P<0.001 vs. H/R group. H/R, hypoxia/reoxygenation; CA, carnosic acid; MMP, mitochondrial membrane potential.

extents (Fig. 6). In particular, the experimental group with 10 μM carnosic acid treatment exhibited $\sim 80\%$ of the fluorescence intensity of the control group. These results revealed that the collapse of MMP in hypoxia/reoxygenation-treated H9c2 cardiomyocytes was relieved by carnosic acid.

Carnosic acid relieves the opening of mPTP in H9c2 cardiomyocytes induced by hypoxia/reoxygenation. The mPTP opening was assessed based on the fluorescence intensity of mitochondrial free calcein. As shown in Fig. 7, following induction of hypoxia/reoxygenation, the fluorescence intensity of mitochondrial calcein in the H/R group was close to half that of the control group ($53.71 \pm 6.49\%$; $P < 0.001$), which revealed the opening of the mPTP. Upon exposure with increasing carnosic acid doses, the opening of the mPTP was ameliorated in a dose-dependent manner, as indicated by the reduced fluorescence intensity of mitochondrial free calcein. Treatment of 10 μM carnosic acid resulted in a fluorescence intensity at $81.74 \pm 6.40\%$ of the control group value ($P < 0.001$).

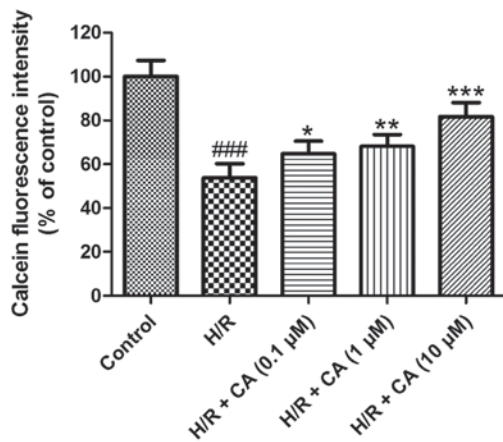


Figure 7. Effect of carnosic acid on mPTP opening, according to the fluorescence intensity of calcein (n=6). ###P<0.001 vs. control group; *P<0.05, **P<0.01 and ***P<0.001 vs. H/R group. H/R, hypoxia/reoxygenation; CA, carnosic acid; mPTP, mitochondrial permeability transition pore.

These results implied that carnosic acid inhibited the mPTP opening that was induced by hypoxia/reoxygenation.

Carnosic acid diminishes caspase-3 activity and affects the expression levels of caspase-3, Bcl-2 and Bax. Caspase-3 is an executioner enzyme in apoptosis and is activated through hydrolysis. The results of the colorimetric assay revealed that the activity of caspase-3 was markedly enhanced in H9c2 cardiomyocytes following treatment of hypoxia/reoxygenation. However, when pretreatment with carnosic acid was performed, the activity of caspase-3 was significantly inhibited (Fig. 8A). Western blot analysis also demonstrated that hypoxia/reoxygenation upregulated the expression of caspase-3, while carnosic acid treatment suppressed this expression in a dose-dependent manner (Fig. 8B). In addition, western blot analysis indicated the expression changes of Bcl-2 and Bax, two proteins associated with apoptosis. Hypoxia/reoxygenation downregulated the expression of Bcl-2 and upregulated Bax expression. On the contrary, different concentrations of carnosic acid resulted in the upregulation of Bcl-2 and downregulation of Bax (Fig. 8B).

Discussion

Myocardial ischemia and reperfusion injury is the major cause of cardiomyocyte apoptosis in myocardial infarction (20). Timely reperfusion disrupts the redox homeostasis and accumulates excessive ROS (3). As the major site of ROS production, the function of mitochondria, including MMP and mPTP, is then severely affected; thus, myocardial cells undergo apoptosis via the mitochondria-mediated pathway (21,22). In addition, during ischemia and reperfusion, the cytosolic calcium overloads and interacts with oxidative stress, which accentuates the dysfunction of the mitochondria via the collapse of MMP and mPTP opening (2,3).

As a member of the cysteinyl aspartate specific protease family, caspase-3 serves a crucial role in apoptosis. Cleavage at specific sites will activate caspase-3 and promote cell apoptosis (23,24). Bcl-2 and Bax are members of the Bcl-2 protein family that are involved in mitochondria-mediated apoptosis.

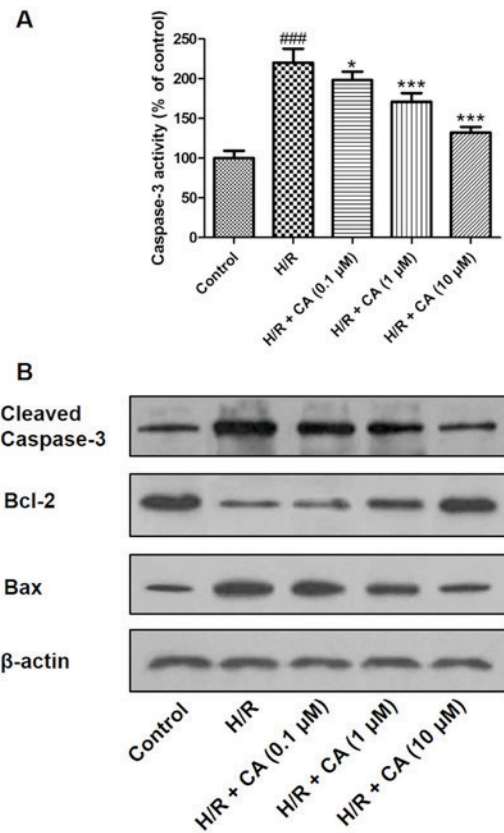


Figure 8. Effect of carnosic acid on the apoptosis of H9c2 cardiomyocytes injured by hypoxia/reoxygenation, examined by western blot analysis. (A) Activity of caspase-3 in H9c2 cardiomyocytes; (B) western blot analysis of the expression levels of cleaved caspase-3, Bcl-2 and Bax (n=6). ###P<0.001 vs. control group; *P<0.05 and ***P<0.001 vs. H/R group. H/R, hypoxia/reoxygenation; CA, carnosic acid; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein.

Bcl-2 inhibits apoptosis and suppresses the activation of caspase-3, while Bax enhances the cell apoptosis (25).

In the discovery of novel therapeutics for myocardial ischemia and reperfusion injury, various natural phytochemicals present promising protection via various pathways. Berberine displayed the protective effects through activating janus kinase 2/signal transducer and activator of transcription 3 signaling and attenuating endoplasmic reticulum stress (5). Furthermore, tanshinone IIA can attenuate the injury by activating the phosphoinositide 3-kinase/Akt/ mammalian target of rapamycin signaling pathway (6). Previous findings have suggested that gypenoside protects the cardiomyocytes against the injury via the mitogen-activated protein kinase-mediated nuclear factor- κ B pathway (26). In addition, as the convergent pathway of final ischemia and reperfusion injury, mitochondria-mediated apoptosis has been demonstrated to be involved in protective effects of most phytochemicals, including clematichinenoside (27) and ilexosaponin A (28).

In the present study, carnosic acid was observed to improve the cell viability and leakage of LDH in H9c2 cardiomyocytes injured by hypoxia/reoxygenation. Further studies have reported that carnosic acid attenuated the overproduction of intracellular ROS, as well as the calcium overload, which reveals that the cardioprotective effect of carnosic acid is associated with the mitochondria-mediated apoptosis pathway.

The present experimental results demonstrated that carnosic acid improved the dysfunction of mitochondria in H9c2 cardiomyocytes through suppressing the collapse of MMP and the mPTP opening, which are pivotal events in apoptosis. At the same time, carnosic acid directly inhibited the apoptosis of H9c2 cardiomyocytes injured by hypoxia/reoxygenation via downregulation of Caspase-3 and Bax, and upregulation of Bcl-2.

As the major phytochemicals in the genera of *Rosmarinus* and *Salvia*, carnosic acid possesses various beneficial bioactivities (11). Previous investigation has revealed carnosic acid may attenuate the isoproterenol-induced myocardial injury in a mouse model through preventing oxidative stress and apoptosis (17). In the present study, the investigations performed further elucidated the protective effects of carnosic acid on myocardial injury *in vitro*, which is reported in H9c2 cardiomyocytes for the first time.

In conclusion, the results of the current study revealed the cardioprotective effects of carnosic acid and the potential underlying mechanisms *in vitro*. These findings provided evidence for further evaluations *in vivo* that may assist in the development of novel therapeutic approaches for myocardial infarction.

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