

Original Article

Alleviation of doxorubicin-induced cardiotoxicity by Hong Huang decoction may involve a reduction in myocardial oxidative stress and activation of Akt/FoxO3a pathways

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Abstract: Doxorubicin (DOX)-induced cardiotoxicity limits the use of DOX as an antitumor drug. This study investigated whether Hong Huang decoction (HHD) inhibits DOX-induced cardiotoxicity. Thirty Balb/c mice were divided into three groups: control, DOX (3 mg/kg i.p., 30 days), and HHD+DOX (DOX plus 9 g/kg HHD by oral gavage). Myocardial enzyme levels, histologic changes, apoptosis (TUNEL assay), and protein expression of cleaved (c)-caspase-3, caspase-3, and Bcl-2 (Western blotting) were assessed. H9c2 cells were assigned to control, DOX, HHD, and HHD+DOX groups. H9c2 cell growth (MTT assay), apoptosis (TUNEL assay), reactive oxygen species (ROS) levels, and expression of c-caspase-3, caspase-3, Bcl-2, p-FoxO3a, and p-Akt (Western blotting) were measured. Regarding *in vivo* experiments, the DOX+HHD group had lower levels of myocardial enzymes ($P < 0.05$), less marked histologic changes, lower apoptosis rates, and higher c-caspase-3 levels than the DOX group ($P < 0.01$). IC_{50} for inhibition of H9c2 cell growth by DOX in the absence and presence of HHD was $3.03 \pm 0.11 \mu\text{M}$ and $7.07 \pm 0.48 \mu\text{M}$, respectively. In H9c2 cells, HHD prevented DOX-induced increase of c-caspase-3/caspase-3 ratio and decrease in Bcl-2 levels ($P < 0.01$). H9c2 cell apoptosis rates were lower in the DOX+HHD group than in the DOX group ($30.2 \pm 2.5\%$ vs. $42.6 \pm 5.2\%$, $P < 0.05$). HHD inhibited DOX-induced increase in ROS levels in H9c2 cells ($P < 0.01$). DOX-induced reductions in p-FoxO3a and p-Akt (S473) protein levels were attenuated by HHD and by the antioxidant, NaHS ($P < 0.05$). HHD protected against DOX-induced cardiotoxicity, *in vitro* and *in vivo*, possibly by reducing oxidative stress and activating Akt/FoxO3a signaling.

Keywords: Hong Huang decoction (HHD), doxorubicin, cardiotoxicity, oxidative stress, Akt, FoxO3a

Introduction

Cancer and cardiovascular disease are the two main causes of mortality worldwide [1]. Doxorubicin (DOX) is an anthracycline-based anti-tumor drug used in many clinical chemotherapy regimens [2]. However, cumulative doses of DOX can cause myocardial damage, including cardiomyopathy and heart failure. This damage is associated with poor prognosis [3-5]. Development of an effective drug to prevent and treat cardiac toxicity induced by DOX would greatly benefit patients undergoing chemotherapy for cancer.

The anticancer effects of DOX are thought to include intercalation into DNA to interfere with protein synthesis and inhibition of topoisomerase II to impair DNA repair. These actions are

also believed to contribute to the cardiotoxicity of DOX [3, 6]. For example, topoisomerase II-beta has been implicated in mediating DOX-induced DNA double-strand breaks in cardiac cells [7] and activating DNA response genes that initiate apoptosis pathways [8]. However, an additional mechanism thought to play an important role in DOX-induced cardiotoxicity is the production of reactive oxygen species (ROS) [8, 9]. Oxidative stress occurs when a cellular imbalance develops between levels of ROS (such as free radicals) and antioxidant mechanisms. Oxidative stress causes myocardial injury and has been implicated in several cardiovascular diseases [9-14]. DOX can be activated by mitochondrial complex I into a more reactive semiquinone derivative that increases oxidative stress, causing oxidative damage to mitochondria [9]. Indeed, DOX has been shown to

enhance lipid peroxidation (thus damaging cell membranes), cause lesions in mitochondrial DNA, and induce free-radical-associated mitochondrial dysfunction [15]. The heart may be particularly susceptible to oxidative stress because normal myocardium contains antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, at lower levels than those found in other tissues [9, 16, 17].

Forkhead homeobox type O (FoxO) transcription factors, including FoxO1, FoxO3a and FoxO4, are critical mediators of cellular responses to oxidative stress. FoxO3a is a particularly important transcription factor because it is involved in not only the response to oxidative stress but also regulation of apoptosis-related genes [18, 19]. Phosphorylation of FoxO3a plays an important role in the modulation of many cellular functions. If phosphorylation of FoxO3a is reduced, nuclear localization of FoxO3a is promoted, leading to cell apoptosis [20]. FoxO3a can be phosphorylated by a variety of kinases, including protein kinase B (Akt) [21]. Activation of Akt can enhance phosphorylation of FoxO3a (at site S253), which retains FoxO3a in the cytoplasm and reduces its transcriptional activity, thereby inhibiting apoptosis. DOX has been demonstrated to downregulate FoxO3a levels in mouse hearts [22] and reduce levels of phosphorylated FoxO3a (p-FoxO3a) in H9c2 cardiac cells [23]. Furthermore, DOX has also been shown to enhance nuclear expression of FoxO3a in H9c2 cells [24]. Interestingly, exogenous hydrogen sulfide (H₂S) has been reported to attenuate DOX-induced toxicity in H9c2 cardiac cells through activation of phosphoinositide 3-kinase (PI3K)/Akt/FoxO3a pathways [25]. In addition, vitexin (an extract from hawthorn leaves) has been demonstrated to suppress DOX-induced oxidative stress, inflammation, apoptosis, and myocardial damage via a mechanism that may involve increased cellular expression of p-FoxO3a [26]. Thus, the Akt/FoxO3a signaling pathway may be a novel target for development of drugs to reduce DOX-induced cardiotoxicity.

Hong Huang decoction (HHD), a Chinese herb formula, contains *Astragalus membranaceus*, rhubarb, turmeric, and *Rhodiola rosea*. A previous study found that HHD exerted a protective effect in patients with breast cancer during DOX-based chemotherapy [27]. Of the various constituents of HHD, *Astragalus polysaccha-*

rides have been demonstrated to exert antioxidant effects and protect cells from apoptosis caused by oxidative stress [28]. *Astragalus polysaccharides* have also been shown to attenuate DOX-induced ROS production and cardiomyocyte apoptosis via a mechanism that involves reduced activation of p38 signaling pathways [29]. Furthermore, Astragaloside IV has been found to inhibit DOX-induced mitochondrial ROS production, mitochondrial damage, and activation of the mitochondrial apoptotic pathway via a mechanism that involves stimulation of PI3K/Akt pathways [30]. Sali-droside, an active component of *Rhodiola*, has been reported to protect heart cells against DOX-induced toxicity by attenuating oxidative stress and inhibiting apoptosis [31]. Curcumin, a component of turmeric, has been suggested to protect against DOX-induced cardiotoxicity [32, 33], although not all studies have agreed [34].

This present study hypothesized that HHD protects against DOX-induced cardiotoxicity by inhibiting oxidative stress and activating Akt/FoxO3a pathways. Therefore, the aim of the present study was to investigate whether HHD attenuated DOX-induced cardiotoxicity, *in vitro* (H9c2 cells) and *in vivo* (in mice), and to examine whether any such effects were associated with reduced levels of oxidative stress and enhanced expression of p-Akt and p-FoxO3a.

Materials and methods

Mice

Protocols for all animal experiments were reviewed and approved by the Institutional Review Board of Animal Research at the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine, Nanjing, China. Female BALB/c mice (6-8 weeks old, weighing 20-25 g) were purchased from the Pharmacology Laboratory at the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine. These animals were maintained in the housing facility under controlled environmental conditions (12/12-hour light/dark cycle).

Preparation of HHD

Astragalus membranaceus (30 g), rhubarb (6 g), turmeric (10 g), and *Rhodiola* (10 g) were acquired from Jiangsu Province Hospital of Traditional Chinese Medicine (Nanjing, China).

These herbs were blended in 1000 mL of double-distilled water (1:10, w/v) for 1 hour and then heated to 100°C for 2 hours to concentrate the sample to a final volume of 50 mL (0.92 g/mL) [35, 36]. After cooling, HHD was stored at 4°C.

In vivo experiments

Mice were randomly divided into three groups ($n = 10$ per group): control, DOX, and DOX+HHD. Mice in the control group received daily oral gavage of normal saline for 25 days and intraperitoneal injections of normal saline on days 5, 10, 15, 20 and 25. Animals in the DOX group received intraperitoneal injections of DOX (3 mg/kg, dissolved in normal saline; no: NSC-123127, Selleck Chemicals, Houston, TX, USA) on days 5, 10, 15, 20 and 25 to obtain a cumulative dose of 15 mg/kg over 25 days [37]. Mice in the DOX+HHD group received intraperitoneal injections of DOX (as with the DOX group) plus daily oral gavage of HHD (9 g/kg). The animals were weighed every 7 days.

Measurement of plasma levels of myocardial enzymes

Blood samples were obtained from the orbit of each mouse on day 30. Blood was centrifuged at 5000 rpm for 3 minutes to separate the plasma. Plasma levels of aspartate aminotransferase (AST), creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH) were measured with a biochemical analyzer (P800, KeyGen Biotech, Nanjing, China).

Histology

Mice were killed by cervical dislocation on day 30 and the hearts were removed and weighed. The left ventricle of the heart was isolated by dissection, fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E), using standard techniques, and viewed using light microscopy [38, 39].

Culture of rat embryonic H9c2 cardiomyocytes

H9c2 cells (KeyGen Biotech) were maintained in complete Dulbecco's Modified Eagle Medium with 10% (v/v) heat-inactivated fetal bovine serum. All cells were maintained at

37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell growth assay

The effects of HHD on cell growth were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation and Cytotoxicity Assay Kit (KeyGen Biotech). Briefly, H9c2 cells in 96-well plates (5,000 cells/well) were incubated for 24 hours with HHD (0-5 µM) in the absence and presence of DOX (2 mg/mL). Next, 10 µL MTT was added to each well and the plates were incubated for 4 hours at 37°C in 5% CO₂ and in the dark. Dimethyl sulfoxide was added to dissolve the formazan crystals. Absorbance at 570 nm was measured using a microplate reader (BioTek Instruments, Winooski, VT, USA).

ROS assay

H9c2 cells (10⁵ cells/well) were cultured for 24 hours in the presence of HHD (2 mg/mL) and/or DOX (1 µM, chosen based on the MTT assay experiments). They were then stained using a ROS kit (Beyotime Institute of Biotechnology, Shanghai, China), in accordance with manufacturer protocol. The cells were imaged using a laser confocal microscope (excitation wavelength, 488 nm; emission wavelength, 525 nm). ROS levels were calculated from the average fluorescence intensity using ImageJ software (National Institutes of Health, Rockville, MD, USA).

Measurement of cell apoptosis

Cell apoptosis in mouse left ventricles and H9c2 cells was measured using TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) assay. TUNEL staining of sections of mouse left ventricles (prepared as described above for H&E staining) was carried out using the TUNEL Cell Apoptosis Detection Kit (Beyotime Institute of Biotechnology). Degree of apoptosis was calculated from 10-15 random fields (400 × magnification). H9c2 cells were fixed in 4% paraformaldehyde, rinsed with phosphate-buffered saline (PBS), and permeabilized with 0.1% Triton X-100 for FITC end-labeling of fragmented DNA of apoptotic H9c2 cells using the TUNEL Cell Apoptosis Detection Kit (Beyotime Institute of Biotechnology). After washing twice with PBS,

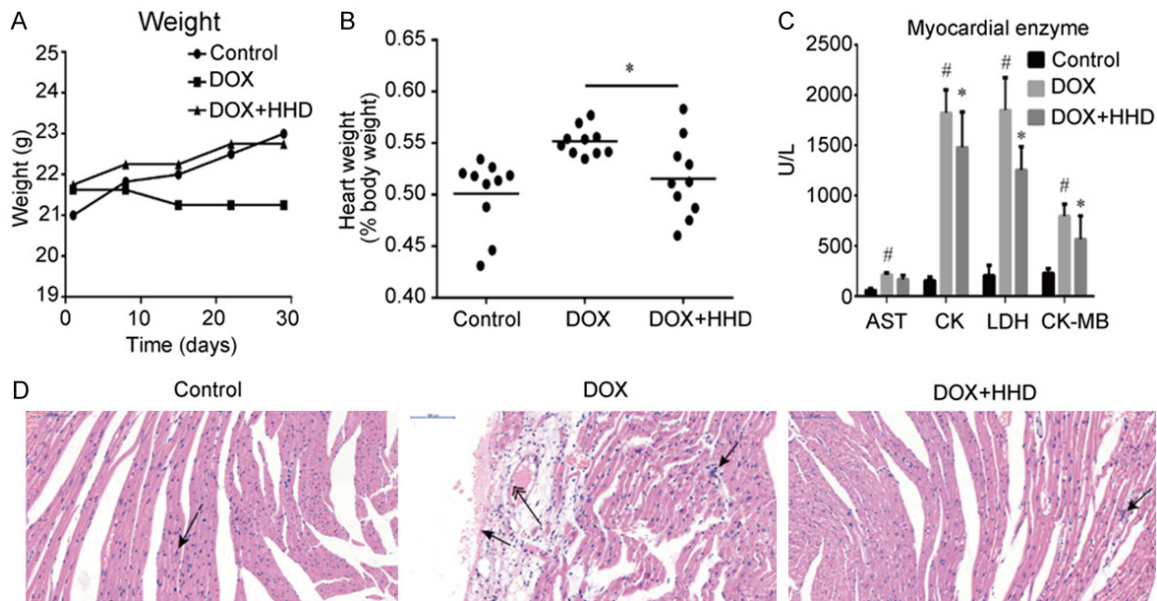


Figure 1. Hong Huang decoction (HHD) attenuated doxorubicin (DOX)-induced cardiomyopathy *in vivo*. A. Mean body weight of the mice in each group ($n = 10$ per group). B. Scatter plot showing the heart weights (expressed as % body weight) of all the mice in each group ($n = 10$ per group). $*P < 0.01$ vs. DOX group. C. Serum levels of aspartate aminotransferase (AST), creatine kinase (CK), creatine kinase isoenzyme MB (CK-MB), and lactate dehydrogenase (LDH). $\#P < 0.05$ vs. control group; $*P < 0.01$ vs. DOX group. D. Representative images for each group showing sections of the left ventricle stained with hematoxylin and eosin ($\times 200$). The DOX group showed focal myocytolysis and necrosis (arrows); note the congested blood vessels (double-headed arrow). The DOX+HHD group exhibited focal myocytolysis (arrow) to a much smaller extent.

cells were stained with 30 μL 4',6-diamidino-2-phenylindole (DAPI). TUNEL-positive cells were imaged under a fluorescence microscope at excitation and emission wavelengths of 488 and 530 nm, respectively.

Western blot analysis

After removal of the left ventricle (used for histology and TUNEL assay), the remaining mouse cardiac tissue was homogenized in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific China, Shanghai, China) for extraction of proteins. Proteins (20 $\mu\text{g}/\text{lane}$) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin in Tris-buffered saline-Tween 20 (TBST, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. Primary antibodies (Cell Signaling Technology, Beverly, CA, USA) used were: rabbit cleaved caspase-3 (c-caspase-3; 1:1000, lot No.: 9664), rabbit caspase-3 (1:1000, lot No.: 9665), and rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:2,000, lot No.: 5174).

Membranes were then washed three times with TBST for 5 minutes and incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:1,000). After washing four times with TBST for 5 minutes, the bands were visualized using an electrochemiluminescence detection kit (Millipore, Billerica, MA, USA). GAPDH served as a loading control for densitometry normalization. Intensities of the Western blot bands were analyzed using ImageJ software.

H9c2 cells were administered 2 mg/mL HHD or/and 1 μM DOX for 24 hours and cell lysates were then prepared. Western blotting was performed, as above, using the following primary antibodies (Cell Signaling Technology): rabbit p-FoxO3a (S253) (1:1000, lot No.: 9466), rabbit FoxO3a (1:1000, lot No.: 12829), rabbit p-Akt (s-473, lot No.: 4046), rabbit Akt (lot No.: 4685), rabbit c-caspase-3 (1:1000, lot No.: 9664), rabbit caspase-3 (1:1000, lot No.: 9665), and rabbit β -actin (1:2,000, lot No.: 4970). The secondary antibody was an HRP-conjugated anti-rabbit secondary antibody (1:1,000). β -actin served as the loading control.

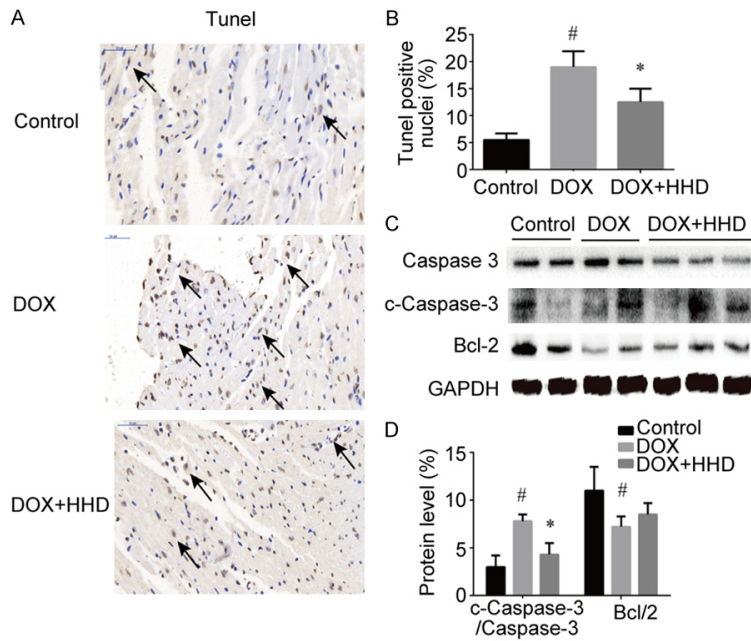


Figure 2. Hong Huang decoction (HHD) reduced doxorubicin (DOX)-induced myocardial apoptosis *in vivo*. A. Representative images for each group showing sections of myocardium subjected to the TUNEL assay ($\times 400$). Arrows: myocardial apoptosis. B. The proportion of TUNEL-positive nuclei in each group. $\#P < 0.05$ vs. control group; $*P < 0.01$ vs. DOX group. C. Western blot images showing protein expression of caspase-3, cleaved caspase-3 (c-caspase-3), Bcl-2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, used as the internal control). D. Mean data quantified from Western blot experiments showing c-caspase-3/caspase-3 ratio, and Bcl-2 level (relative to that of GAPDH) in each experimental group. $\#P < 0.05$ vs. control group; $*P < 0.01$ vs. DOX group.

Statistical analysis

Data were analyzed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and are expressed as mean $\pm$ standard error of the mean (SEM) or standard deviation (SD). Statistical comparisons were made using one-way analysis of variance (ANOVA) and Fisher's least significant difference (LSD) post-hoc test. $P < 0.05$ is considered statistically significant.

Results

HHD reduced doxorubicin-induced cardiomyopathy *in vivo*

Over the course of the 30-day experimental period, the average weight of mice increased progressively from 21.00 ± 1.67 g to 23.00 ± 1.26 g in the control group but decreased slightly in the DOX group from 21.63 ± 1.06 g to 21.25 ± 1.28 g (Figure 1A). However, mice in the DOX+HHD group exhibited an increase in

weight from 21.75 ± 1.66 g to 22.75 ± 1.28 g (Figure 1A), indicating that HHD was able to attenuate the detrimental effects of DOX on animal growth. Thirty days after initiation of treatment, heart weight (as a % of body weight) was numerically higher in the DOX group than the control group (Figure 1B). Furthermore, heart weight was significantly lower in the DOX+HHD group than in the DOX group ($P < 0.05$; Figure 1B). To confirm whether DOX induced myocardial damage, serum levels of myocardial enzymes were measured. The DOX group exhibited significantly elevated levels of AST, CK, LDH, and CK-MB compared with control mice (all $P < 0.05$; Figure 1C). However, compared with the DOX group, DOX+HHD group showed significantly lower levels of AST (166 ± 44 vs. 212 ± 23 U/L; $P < 0.01$), CK (1480 ± 355 vs. 1823 ± 232 U/L, $P < 0.01$), LDH (1256 ± 233 vs. 1852 ± 322 U/L; $P < 0.01$), and CK-MB (567 ± 233 vs. 795 ± 121 U/L; $P < 0.01$). In the control group, sections of left ventricles stained with H&E showed no evidence of inflammation or myocardial fibrosis (Figure 1D). In contrast, the DOX group exhibited myocardial necrosis, inflammatory cell infiltration, myocardial edema, and hemorrhaging in the ventricular wall (Figure 1D). Although a small amount of inflammatory cell infiltration was observed in the DOX+HHD group, there was no myocardial necrosis, myocardial edema, or hemorrhaging (Figure 1D). Taken together, these data suggest that HHD alleviated DOX-induced cardiomyopathy.

HHD inhibited DOX-induced myocardial apoptosis *in vivo*

TUNEL assay was applied to sections of mouse myocardium to detect apoptosis (Figure 2A). Rates of apoptosis were significantly higher in the DOX group than the control group ($P < 0.05$; Figure 2B), consistent with DOX-induced car-

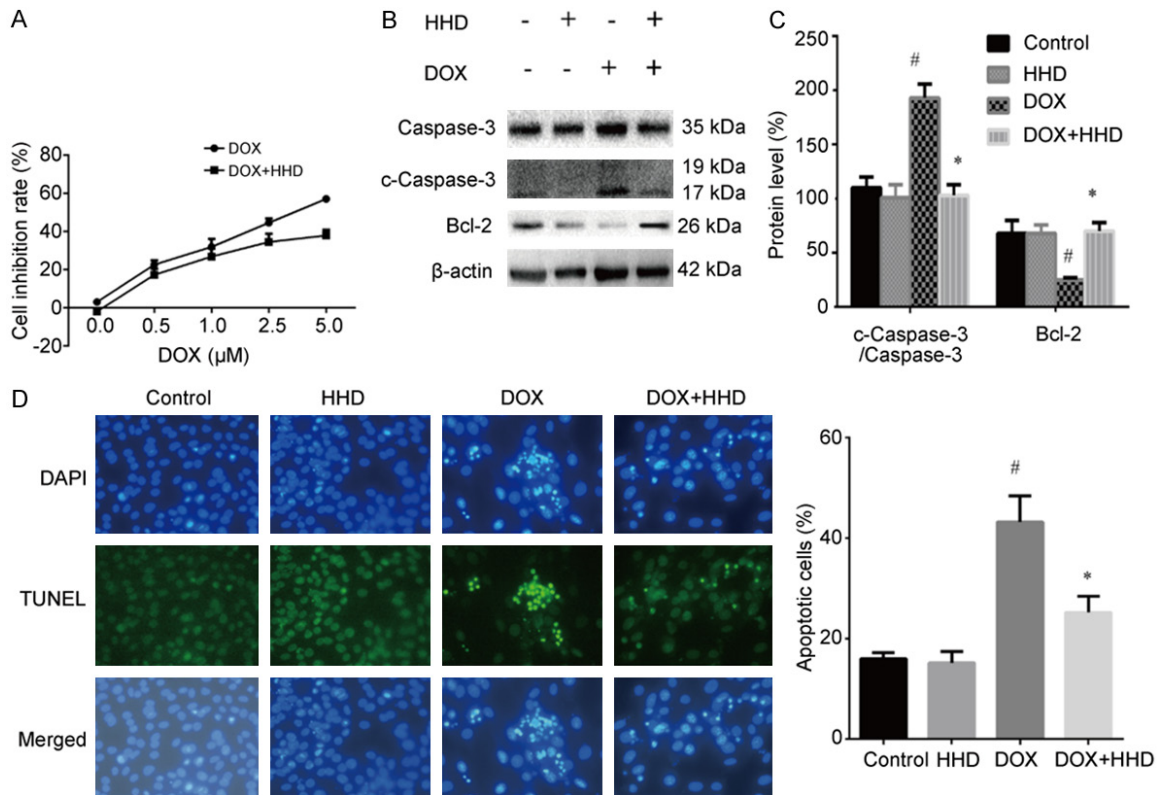


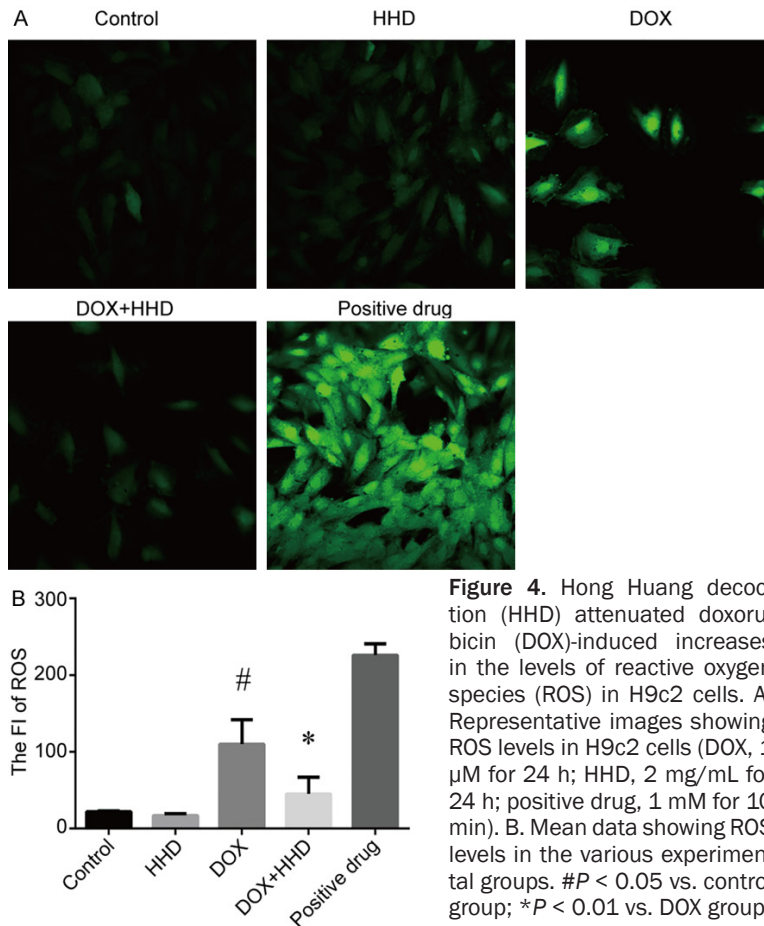
Figure 3. Hong Huang decoction (HHD) reduced doxorubicin (DOX)-induced myocardial apoptosis in H9c2 cells. **A.** Concentration-dependent inhibition of H9c2 cell growth (assessed using the MTT assay) by DOX in the absence and presence of HHD (2 mg/mL). **B.** Representative Western blots showing the protein expression of caspase-3, cleaved caspase-3 (c-caspase-3), Bcl-2, and β -actin (used as the internal control) in each experimental group (DOX, 1 μ M; HHD, 2 mg/mL; for 24 h). **C.** Mean data quantified from Western blot experiments showing c-caspase-3/caspase-3 ratio, and Bcl-2 level (relative to that of β -actin) in each experimental group. # $P < 0.05$ vs. control group; * $P < 0.01$ vs. DOX group. **D.** Left: representative fluorescence images of H9c2 cells stained using the TUNEL assay (green) to show apoptosis and DAPI (blue) to identify the nuclei (DOX, 1 μ M; HHD, 2 mg/mL; for 24 h). Right: quantification of apoptosis rate from the TUNEL assay. # $P < 0.05$ vs. control group; * $P < 0.01$ vs. DOX group.

diomyopathy. Notably, apoptosis rates were significantly lower in the DOX+HHD group than the DOX group ($P < 0.01$; **Figure 2B**). Cleaved caspase-3 has been thought to carry out most of the proteolysis that occurs during apoptosis and detection of c-caspase-3 has been considered a reliable marker of cells undergoing apoptosis [40]. Protein levels of c-caspase-3 in mouse myocardium were significantly elevated in the DOX group compared with controls ($P < 0.05$), but this elevation was significantly attenuated by HHD ($P < 0.01$; **Figure 2C, 2D**). Compared with the control group, DOX also downregulated expression of Bcl-2 ($P < 0.05$; **Figure 2C, 2D**), a protein known to inhibit apoptosis [41]. This reduction in Bcl-2 protein expression appeared to be less marked in the DOX+HHD group (**Figure 2C, 2D**), although there were no significant differences between

the DOX and DOX+HHD groups. These data provide good evidence that HHD inhibited DOX-induced myocardial apoptosis *in vivo*.

HHD reduced DOX-induced apoptosis in H9c2 cells *in vitro*

MTT assay was used to measure inhibition of H9c2 cell growth by a range of DOX concentrations (0–5 μ M). The effects of HHD (2 mg/mL) were determined. In the absence of HHD, the concentration of DOX causing 50% inhibition of cell growth (IC_{50} value) at 24 hours was 3.03 ± 0.11 μ M. However, in the presence of HHD, the IC_{50} value for DOX was 7.07 ± 0.48 μ M. (**Figure 3A**), implying that HHD attenuated DOX-induced reduction in H9c2 cell viability. Based on these results, the concentration of DOX used for subsequent experiments was 1 μ M.



levels in H9c2 cells are shown in **Figure 4A**. ROS levels were significantly higher in the DOX group than in the control group ($P < 0.05$; **Figure 4B**). Moreover, ROS levels were significantly lower in the DOX+HHD group than in the DOX group ($P < 0.01$; **Figure 4B**). These results demonstrate that HHD was able to reduce oxidative stress caused by DOX.

HHD activated Akt/FoxO3a signaling pathways in H9c2 cells in vitro

Treatment of H9c2 cells with DOX resulted in a reduction in levels of p-FoxO3a and p-Akt (S473). Importantly, p-FoxO3a and p-Akt (S473) levels were significantly higher in the DOX+HHD group than in the DOX group ($P < 0.01$; **Figure 5A**). To gain insight into whether these effects of HHD might be mediated by a reduction in oxidative stress, additional experiments were conducted using sodium hydrogen sulfide (NaHS), an antioxidant reported to reduce DOX-induced apoptosis of H9c2 cells [42]. NaHS was found to exert similar effects to HHD on DOX-induced levels of p-FoxO3a and p-Akt (S473) (**Figure 5B**). These data raise the possibility that the protective effects of HHD on DOX-induced cardiotoxicity may be due, at least in part, to a reduction in oxidative stress and activation of Akt/FoxO3a signaling.

Western blotting was employed to measure levels of apoptosis-related proteins in H9c2 cells after treatment for 24 hours with DOX (1 μ M), HHD (2 mg/mL), or their combination (**Figure 3B**). Compared with the control group, the DOX group showed an increase in c-caspase-3/caspase-3 ratio and a decrease in Bcl-2 levels ($P < 0.05$, **Figure 3C**). These effects of DOX were prevented by co-administration of HHD ($P < 0.01$, **Figure 3C**). HHD alone was without significant effect (**Figure 3C**). In further experiments, rates of H9c2 cell apoptosis were measured by TUNEL assay. Apoptotic cell rates were significantly lower in the DOX group than the DOX+HHD group ($30.2 \pm 2.5\%$ vs. $42.6 \pm 5.2\%$; $P < 0.05$; **Figure 3D**). HHD alone had little or no effect (**Figure 3D**). These data indicate that HHD could protect against DOX-induced apoptosis of H9c2 cells.

HHD reduced oxidative stress caused by DOX in H9c2 cells in vitro

Representative confocal microscopy images from each experimental group showing ROS

Discussion

One previous study found that HHD exerted protective effects in patients with breast cancer during DOX-based chemotherapy [27]. A notable finding of the present study was that HHD was able to attenuate DOX-induced cardiotoxicity in mice, *in vivo*, as evidenced from histologic observations and from measurements of serum myocardial enzymes levels, apoptosis rates, and c-caspase-3 levels. In H9c2 cells, the IC_{50} value for growth inhibition by DOX was higher in the presence of HHD than in the absence of HHD. Moreover, HHD prevent-

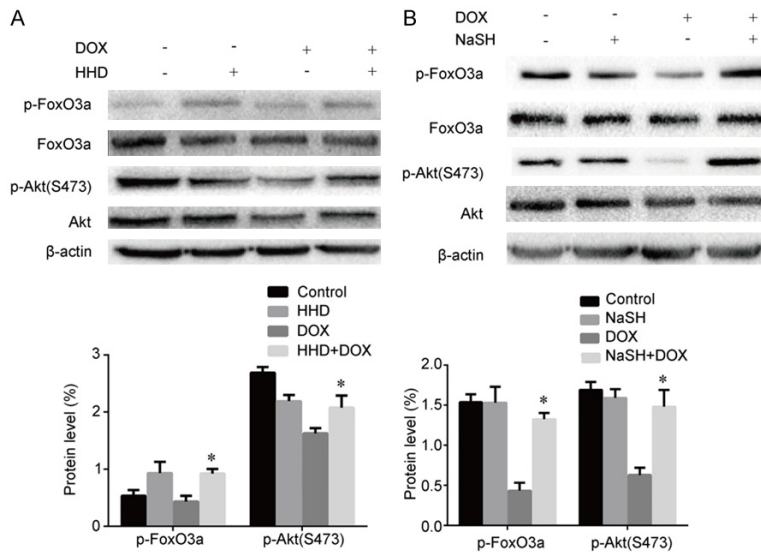


Figure 5. Hong Huang decoction (HHD) activated Akt/FoxO3a signaling pathways in H9c2 cells treated with doxorubicin (DOX). A. Top: representative Western blots showing protein expression of FoxO3a, phosphorylated FoxO3a (p-FoxO3a), Akt, p-Akt, and β -actin (used as the internal control) in H9c2 cells from each experimental group (DOX, 1 μ M; HHD, 2 mg/mL; for 24 h). Bottom: mean data quantified from Western blot experiments showing p-FoxO3a and p-Akt levels (relative to that of β -actin) in each experimental group. B. Top: representative Western blots showing the protein expression of FoxO3a, p-FoxO3a, Akt, p-Akt, and β -actin in H9c2 cells from each experimental group (DOX, 1 μ M; NaHS, 100 μ M; for 24 h). Bottom: mean data quantified from the Western blot experiments showing p-FoxO3a and p-Akt levels (relative to that of β -actin) in each experimental group. * $P < 0.05$ vs. DOX group.

ed DOX-induced apoptosis of H9c2 cells and inhibited DOX-induced increase in ROS levels in these cells. Importantly, DOX-induced reductions in p-FoxO3a and p-Akt (S473) protein levels in H9c2 cells were attenuated by HHD and the effects of HHD were mimicked by the antioxidant, NaHS. Taken together, these findings suggest that HHD can protect against DOX-induced cardiotoxicity, *in vitro* and *in vivo*, possibly by reducing oxidative stress and activating Akt/FoxO3a signaling pathways.

Although DOX is an effective anticancer drug, the associated cardiotoxicity is a limiting factor in its clinical use. *In vivo* experiments, in the present study, demonstrated that DOX caused histologic changes in the heart (consistent with myocardial damage) and increased serum cardiac enzyme levels, myocardial c-caspase-3 protein levels, and cardiomyocyte apoptosis rates. In H9c2 cells, DOX also increased c-caspase-3 protein levels and apoptosis rates. These findings are consistent with numerous previous studies of DOX-induced cardiotoxicity, *in vivo* and *in vitro* [22-26, 29-34, 42]. Thus, the

model systems used in the present study are reproducible and suitable for studies concerning DOX-associated cardiotoxicity.

It has been recognized that induction of oxidative stress is an important mechanism contributing to the adverse cardiac effects of DOX [9, 15]. This study observed that treatment of cultured H9c2 cells with DOX resulted in a substantial elevation in cellular levels of ROS, in agreement with previous studies [25, 29-31, 33, 42]. H_2O_2 , known to impose oxidative stress on cells, has been shown to mimic DOX and promote cardiomyocyte apoptosis [25]. Furthermore, the antioxidant H_2S has been reported to inhibit the detrimental actions of DOX on heart cells [25, 42]. The present study found that inhibition of DOX-induced H9c2 cell apoptosis by HHD was associated

with a reduction in ROS levels, suggesting that the beneficial effects of HHD on cell survival may be mediated, at least in part, by suppression of DOX-induced oxidative stress. In agreement with the present data, previous studies of vitexin [26], cilostazol [43], vitamin C [44], allicin [45], *Zingiber officinale* extract [46], and various components of HHD, including Astragalus polysaccharides [28, 29], salidroside [31], and curcumin [32, 33], have found that inhibition of oxidative stress may protect against DOX-induced toxicity. Therefore, based on present findings, this study proposes that inhibition of ROS levels may be an important mechanism contributing to beneficial effects of HHD against DOX-induced cardiotoxicity. Although the present study did not identify specific components of HHD exerting antioxidant effects, it is likely that multiple constituents contribute [28, 29, 31-33].

Several mechanisms have been suggested to underlie the protective effects of agents that reduce oxidative stress in cardiomyocytes exposed to DOX. For example, the beneficial ef-

fects of H₂S in DOX-treated H9c2 cells have been attributed to suppression of p38 mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) 1/2 signaling [25, 47], inhibition of downstream nuclear factor-kappa-B (NF-κB) pathways [47], and suppression of calreticulin expression [48]. Interestingly, reduced activation of p38 signaling has also been implicated in mediating the protective effects of Astragalus polysaccharides against DOX-induced ROS production and apoptosis [29]. However, Akt signaling is another pathway that plays a key role in cell growth and apoptosis. Akt activation has been thought to promote cardiac cell survival by inhibiting apoptosis [49]. Akt can phosphorylate and regulate FoxO3a, a multifunctional transcription factor involved in regulation of differentiation, development, proliferation, apoptosis, and necrosis [50]. FoxO3a can transduce a variety of cellular stimuli, including oxidative stress, resulting in either apoptosis or protective cell growth arrest [51, 52]. DOX has been shown to reduce levels of FoxO3a in mouse hearts [22], decrease levels of p-FoxO3a in H9c2 cells [23], and upregulate nuclear expression of FoxO3a in H9c2 cells [24]. Furthermore, H₂S and vitexin have been suggested to attenuate DOX-induced toxicity in H9c2 cardiac cells by activating FoxO3a pathways [25, 26]. This study found that HHD increased levels of p-Akt and p-FoxO3a, consistent with stimulation of Akt/FoxO3a signaling. Therefore, this present study proposes that inhibition of oxidative stress and activation of the Akt/FoxO3a pathway may be a mechanism that contributes to the protective effects of HHD against DOX-induced cardiotoxicity. Interestingly, the beneficial effects of Astragaloside IV (a component of HHD) have also been attributed to stimulation of PI3K/Akt signaling [30]. Additional studies are necessary to identify the specific components of HHD that inhibit ROS levels and stimulate Akt/FoxO3a signaling in cardiomyocytes treated with DOX.

In conclusion, this study has shown that HHD, a Chinese herbal medicine, protects against DOX-induced cardiotoxicity, *in vitro* and *in vivo*. Furthermore, these actions may be mediated, at least in part, by a reduction in oxidative stress and activation of Akt/FoxO3a signaling pathways. Further studies are necessary to characterize the mechanisms in more detail and establish, definitively, whether HHD is clinically useful as an inhibitor of DOX-induced cardiotoxicity.

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Disclosure of conflict of interest

None.

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