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Original Article

Didymin attenuates doxorubicin-induced cardiotoxicity by inhibiting oxidative stress

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ABSTRACT

Objective: This study was designed to investigate the protective effects of didymin (Did) on doxorubicin (DOX)-induced cardiotoxicity.
Methods: After pretreatment with Did (2, 4, 8 mg/kg intraperitoneal i.p.) for 7 d, the male C57 mice were injected with single dose of DOX (20 mg/kg i.p.). The cardioprotective effect of Did was observed on the 7th day after DOX treatment.
Results: DOX delayed body growth and caused cardiac tissue injury, oxidative stress, and mitochondrial dysfunction. Similar experiments in H9C2 cardiomyocytes showed that DOX reduced cell viability, increased generation of reactive oxygen species (ROS) and fragmentation of DNA, decreased mitochondrial membrane potential, and induced cardiomyocyte apoptosis. However, all of these adverse effects were suppressed by Did pretreatment. Did increased protein expression of glutamate-*L*-cysteine ligase catalytic subunit (GCL), heme oxygenase 1 (HO-1), and nuclear factor erythroid 2-related factor 2 (Nrf2). Besides, Did also induced activation of PI3K/AKT.
Conclusion: These findings indicated Did prevented DOX-induced cardiac injury and apoptosis via activating PI3K/AKT/Nrf2 signaling pathway.

1. Introduction

As a potent anti-tumor agent, Doxorubicin (DOX) has been widely used for the treatment of different types of cancer, such as leukemia, lymphomas, and breast cancer (Benjanuwattra et al., 2020). However, DOX has a dose-dependent cardiotoxicity and often results in left ventricular dysfunction, cardiomyopathy and even heart failure in humans, which limited its clinical use in chemotherapy (Mancilla, Iskra, & Aune, 2019). Therefore, novel strategies and compounds to reduce DOX-induced cardiotoxicity are necessary. DOX induces oxidative stress and mitochondria-mediated apoptosis in cardiomyocytes, which is mediated by high levels of reactive oxygen species (ROS), contribute to the development of heart failure (Abdullah et al., 2019). Study shows that antioxidants treatment protects against DOX-induced cardiomyocytes injury (Chen et al., 2015).

Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important transcription factor. Under normal conditions, a ubiquitin ligase actin binding protein, Kelch-like ECH-associated protein 1 (Keap1) binds with Nrf2 in the cytoplasm and promotes its proteasomal degradation. Cysteine residues on Keap1 are oxidized when exposed to oxidants, and prevents binding to Nrf2 (Dai et al., 2020). The released Nrf2 translocates into the nucleus and combines with antioxidant-response elements (AREs) to regulate the expression of target genes, such as γ-glutamyl cysteine synthetase (GCS), heme oxygenase 1 (HO-1), NAD(P)H quinone reductase (NQO1), sestrin2 (Sesn2), and glutamate-*L*-cysteine ligase catalytic subunit (GCL). These proteases can scavenge free radicals and protect our organism from oxidative stress (Yao et al., 2020). Nrf2 signaling is involved in DOX-induced apoptosis of cardiomyocytes (Zhao et al., 2020).

Flavonoids are naturally occurring polyphenolic compounds and have a wide range of biological activities (Navarro-Hortal et al., 2020). Didymin (Did) is a flavonoid that is high in concentration in citrus fruits, such as oranges, bergamot, mandarin, and lemons (Yao et al., 2018). Did can alleviate hepatic fibrosis and ameliorate CCl4-induced liver injury (Lin et al., 2016); it has anticancer effects and can induce apoptosis in human hepatoma cells and neuroblastoma (Hung et al., 2010, Singhal et al., 2012). However, the protective effect of Did on DOX-induced cardiotoxicity

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has not been studied. In our previous study, we found that total flavonoids from *Clinopodium chinense* (Benth) O. Ktze showed protective effect on myocardial damage induced by Dox treatment (Chen et al., 2015). Did is the most abundant component of the total flavonoids from *C. chinense*. The purpose of our study is to discuss how Did protects against DOX-induced cardiotoxicity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Materials and chemicals

Did was provided by Shanghai Win Herb Medical S & T Development (Shanghai, China, purity > 99%). Primary antibodies against Bax, Bcl-2, Caspase-3/9, GCL, HO-1, Nrf2, Lamin A, AKT, P-AKT, PI3K, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Kits for determining lactate dehydrogenase (LDH), aspartate aminotransferase (AST), creatine kinase isoenzyme (CK), malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-PX) were obtained from Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits of HO-1 and GCL were obtained from Wuhao Trade (Shang Hai, China). The ROS detection kit was obtained from Invitrogen (California, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals and experimental protocols

Male BALB/c mice (6–8 weeks old) used in this study were obtained from Vital River Laboratory Animal Technology (Beijing, China). The mice were maintained under standard environmental conditions (room temperature at 25 ± 1 °C and humidity of 60% with 12 h light/dark cycle). A total of 80 mice were randomly divided into the following groups: (1) control group: mice were injected with normal saline i.p. (solvent for DOX and Did); (2) DOX group: mice were treated with normal saline i.p. every day for one week, and then treated with DOX i.p. with single dose of 20 mg/kg. (3) Did + DOX group: mice were treated with Did at doses of 2, 4, and 8 mg/kg i.p. every day for one week followed by DOX at 20 mg/kg.

The body weight of mice was measured on 7th days after the first administration of DOX. Echocardiographic measurements were carried out to evaluate myocardial systolic function. The serum was collected for testing levels of LDH, CK and AST. The histopathological examination of heart tissues was conducted. Myocardial homogenates were used for measuring activities of MDA, GSH-Px, CAT, and SOD. All experimental procedures and protocols were approved by the Laboratory Animal Ethics Committee of the Institute of Medicinal Plant Development, Peking Union Medical College (No. 20181016).

2.3. Histological studies

Heart tissues were fixed in 4% paraformaldehyde buffer overnight. The ventricular tissues were subsequently embedded in paraffin after dehydration and were serially cut into 4 μm thick sections. HE staining were carried out according to the standard protocols. The structure of hearts was observed by a light microscope (CKX41, 170 Olympus, Tokyo, Japan). Three randomly selected visual fields were used to indicate the histological changes.

2.4. Electron microscopy

The left ventricle was isolated and cut into 1 mm^3 sized pieces and then fixed in phosphate buffered 2.5% glutaraldehyde solution (pH 7.4) immediately. Ultrathin sections were fixed with 1% osmium tetroxide, dehydrated through graded series of ethanol solutions, embedded in Epon™ medium, stained with uranyl acetate and lead citrate, and then observed under H-7600 electron microscope (Hitachi Medical Corp, Tokyo, Japan).

2.5. Cell culture and treatment

H9c2 cells were cultured in DMEM supplemented with 10% (volume percent) fetal bovine serum, 2 mL glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were cultured in a humidified incubator with 95% air and 5% CO_2 at 37 °C. In all experiments, the cells were plated at an appropriate density and incubated for 36 h prior to being subjected to various treatments. The cells were divided into five groups. The control group and the DOX group were treated with DMSO (final concentration was < 0.1%). Three other groups were treated with different doses of Did (5, 10, 20 μg/mL) for 4 h. then DOX group and Did group were treated with 1 μmol/L DOX for 24 h.

2.6. Cell viability analysis

MTT assay was used to evaluate cell viability. Briefly, the cells were incubated in 96-well plates for 36 h. After treatment with Did and DOX, the cells were added with 20 μL MTT solution (1 mg/mL final concentration) at 37 °C for 4 h. Next, the MTT solution was replaced with 100 μL of DMSO to dissolve formazan crystals. After 10 min shaking, optical density was measured at 570 nm on a microplate reader (BioTek, Vermont).

2.7. Detection of LDH, MDA, SOD, GSH-PX, CAT, HO-1 and GCL activities

H9c2 cells were plated in six-well plates. After treatment, the level of LDH in supernatant and activities of MDA, SOD, GSH-PX, and CAT in cells were detected by corresponding kit. ELISA kits were used to measure the expression levels of HO-1, and GCL in supernatant.

2.8. Measurement of intracellular ROS production

The ROS production in cells was analyzed using corresponding kit. Briefly, cells were washed with buffer after treatment, and then ROS detection solution was added. The cells were stained at 37 °C in the dark for 30 min and visualized by fluorescence microscopy (Leica, Heidelberg, Germany).

2.9. Hoechst 33342 staining

Hoechst 33342 staining, which distinguishes apoptotic cells from normal cells on the basis of nuclear chromatin condensation and fragmentation, was used to qualitatively analyze apoptotic cells. H9c2 cells were cultured on cover slips in 24-well plates for 36 h. After treatment, the cells were incubated with 5 mg/mL Hoechst33342 at 37 °C for 15 min and held twice with PBS, and then observed using fluorescence microscopy (Leica, Heidelberg, Germany).

2.10. Measurement of mitochondrial membrane potential ($\Delta\Psi$)

The changes of mitochondrial transmembrane potential were detected by 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazo

lyl-carbocyanine iodide (JC-1). H9c2 cells were cultured on cover slips in 24-well plates. After treatment, H9c2 cells were incubated with JC-1 (1 μM) in dark at 37 °C for 20 min and washed with PBS. The cells labeled with JC-1 were observed under fluorescence microscopy (Leica, Heidelberg, Germany).

2.11. Western blot analysis

Western blot was performed according to protocols used in our lab. Briefly, H9c2 cells were lysed using pre-cooled lysis buffer consisting of 1 mmol/L PMSF and RIPA. The lysates were centrifuged at 12 000 rpm for 15 min at 4 °C and supernatants were collected. The protein concentration was measured using BCA kit. Samples were mixed with SDS loading buffer (4:1) and then boiled at 100 °C for 5 min and stored at − 80 °C until use. Equal amounts of protein fractions were electrophoresed by 12% SDS-PAGE, and then electro-transferred onto PVDF membranes. The membranes were blocked with TBST containing 5% nonfat milk for 2 h at room temperature with gentle shaking. After blocking, the membranes were incubated with primary antibodies at 4 °C overnight, washed thrice with TBST, incubated with secondary antibodies for 2 h at room temperature, and washed again thrice with TBST. Protein band intensities of membranes were developed using ECL kit. The protein expression levels were visualized with Image Lab Software.

2.12. Statistical analysis

Results from at least three independent experiments were expressed as mean ± SD. Statistical comparisons between different groups were measured by using Student'st-test or ANOVA with Prism 5.00 software. Statistical significance was considered at $P < 0.05$.

3. 3.Results

3.1. Did prevents DOX-induced cardiac injury and heart dysfunction in mice

Compared with control group, the body of mice treated with DOX decreased significantly. By contrast, mice subjected to Did pretreatment (2, 4, and 8 mg/kg) showed dose-dependent recovery of body weights (Fig. 1A). Levels of CK, AST, and LDH in mice serum were measured to evaluate the protection of Did on cardiac injury. Did treatment inhibited DOX-induced increase of LDH, CK, AST in a dose dependent significantly (Fig. 1B − D)

DOX administration induced myocardial systolic dysfunction in mice, as shown by the reduction of EF and FS, compared with the results obtained with saline treated mice, which were attenuated by pre-treatment with Did (Fig. 2).

As shown in Fig. 3A, heart tissues of mice in control group had no obvious abnormalities. DOX group displayed severe myocardial damages, characterized by disorganization of myofibrillar arrays and cytoplasmic vacuolization. We further found that DOX induced cytoplasmic vacuolation, myofibrillar loss, chromatin condensation, mitochondrial edema, and cardiomyocyte necrosis in heart tissues of mice using transmission electron microscopy (Fig. 3B). However, these pathological changes were alleviated by Pretreatment with Did partially.

3.2. Did enhances antioxidant capacity in cardiac tissues

We measured the oxidative stress-associated parameters in the heart. DOX-induced oxidative stress injury in mice heart tissues, as indicated by decreased SOD, CAT, and GSH-Px activities and

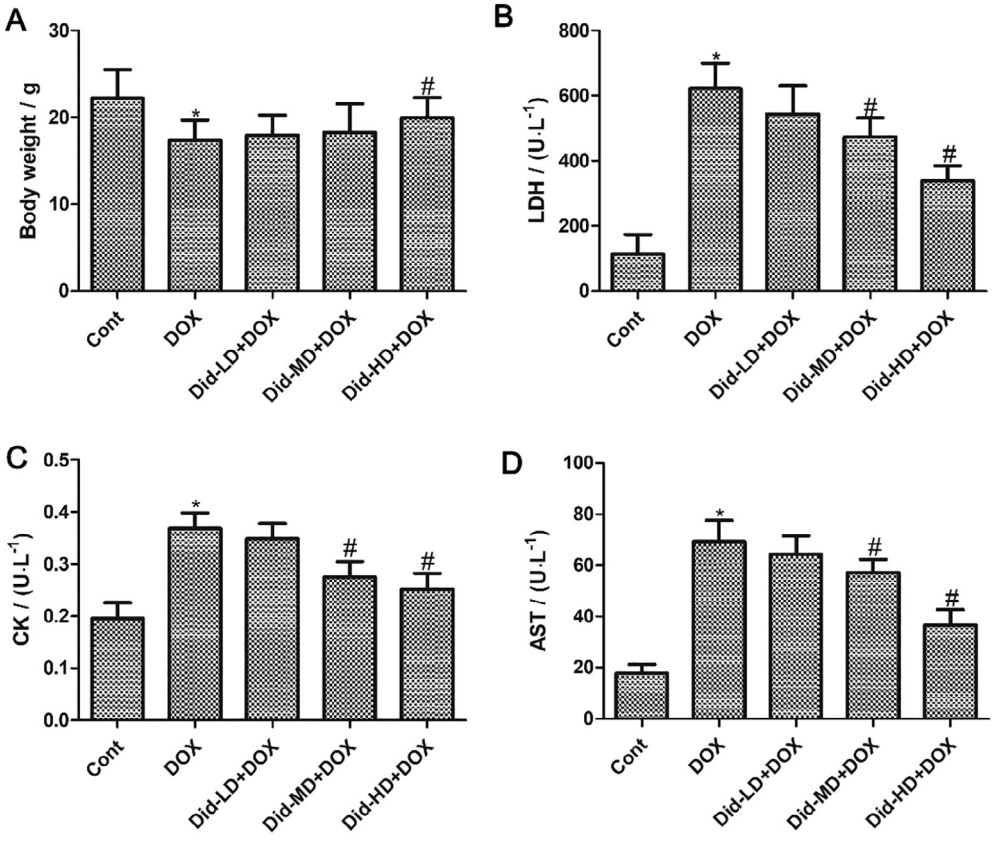

Fig. 1. Effects of Did on DOX-induced myocardial injury *in vivo* (mean ± SD, $n = 10$). Mice were treated with vehicle or DOX with or without Did pretreatment. At day 7, body weight of mice was determined (A); Effects of Did and DOX on AST (B), LDH (C), and CK (D) activities were measured. *$P < 0.05$ *vs* control group, and $^{\#}P < 0.05$ *vs* DOX group. Did-LD + DOX: low-dose Did group (2 mg/kg); Did-MD + DOX: medium-dose Did group (4 mg/kg); Did-HD + DOX: high-dose Did group (8 mg/kg).

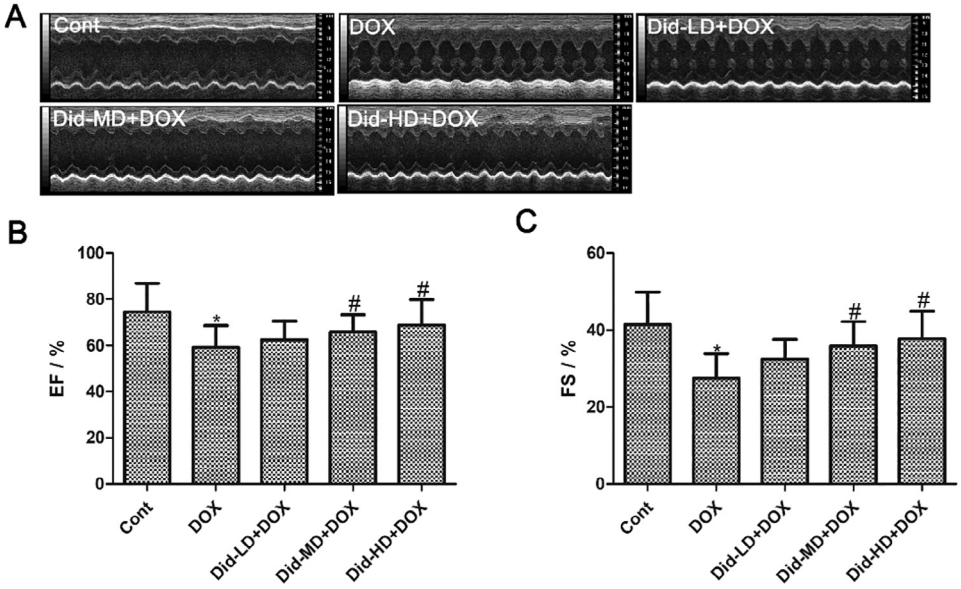

Fig. 2. Effects of Did and DOX on cardiac function. (A) Representative M−mode ECG images are shown. Echocardiography values are expressed as mean ± SD. (B) EF, ejection fraction. (C) FS, fractional shortening. Results are represented as the mean ± SE (n = 6). *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group. Did-LD + DOX: low-dose Did group (2 mg/kg); Did-MD + DOX: medium-dose Did group (4 mg/kg); Did-HD + DOX: high-dose Did group (8 mg/kg).

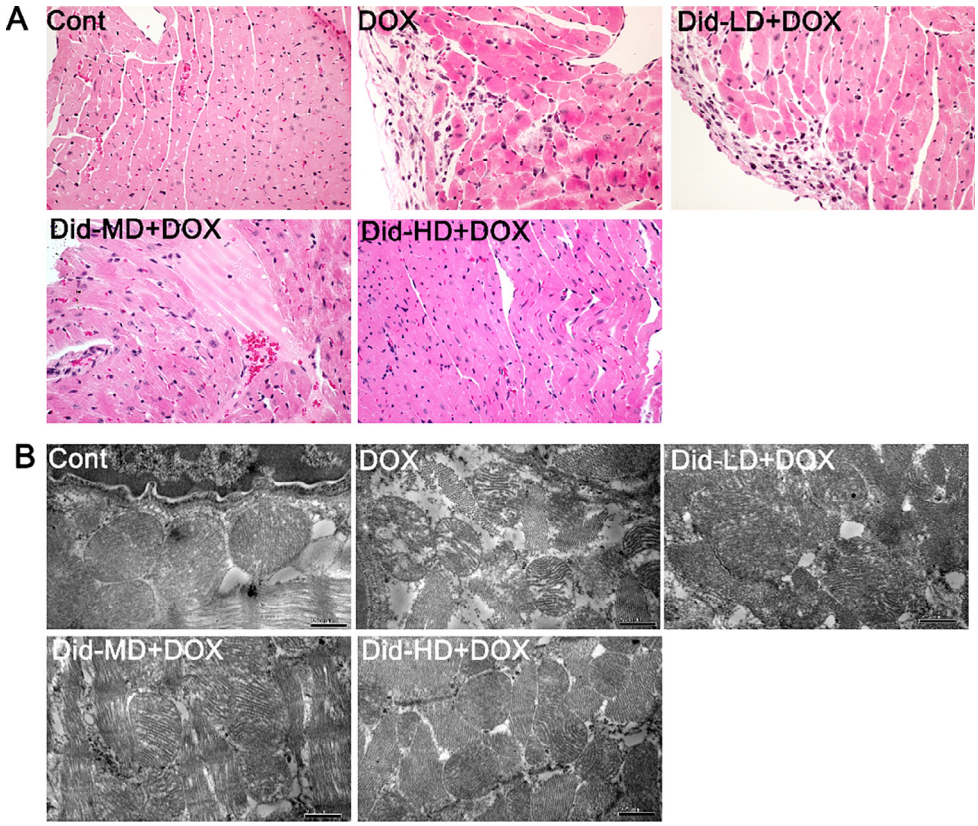

Fig. 3. Effects of Did and DOX on histological changes in mice hearts, as shown by HE staining (A) under electron microscope. Effects of Did and DOX on ultra structure changes in mice hearts observed under transmission electron microscopy (B). Did-LD + DOX: low-dose Did group (2 mg/kg); Did-MD + DOX: medium-dose Did group (4 mg/kg); Did-HD + DOX: high-dose Did group (8 mg/kg).

increased MDA production (Fig. 4), which were ameliorated by Did in a dose dependent.

3.3. Did protects against DOX-induced cytotoxicity in H9c2 cells

The protective effect of Did against DOX-induced injuries in H9c2 Cells was detected. As shown in Fig. 5, DOX significantly reduced cell viability and increased LDH release, whereas pretreatment with Did significantly maintained cell viability and reduced LDH release.

3.4. Did protected against DOX-induced apoptosis in H9c2 cells

The nuclear of H9c2 cells with DOX exposure showed heterogeneous intensity and chromatin condensation, which indicated apoptotic occurrence in cells. Pretreatment with Did for 4 h exhibited a strong anti-apoptotic effect (Fig. 6 A and C). Mitochondrion is an important organelle related to cell energy supply and cellular apoptosis. A decrease in mitochondrial transmembrane potential may trigger a cascade of apoptotic signaling. In our experiment, JC-1 staining showed that Did pretreatment abates DOX-induced decrease in mitochondrial transmembrane potential in H9c2 cells (Fig. 6 B and D).

3.5. Did decreased ROS production and enhanced antioxidant capacity in H9c2 cells

We next detected the potent ROS-scavenging effect of Did. As shown in Fig. 7, compared with control group, DOX treatment increased ROS production significantly in H9c2 cells. However,

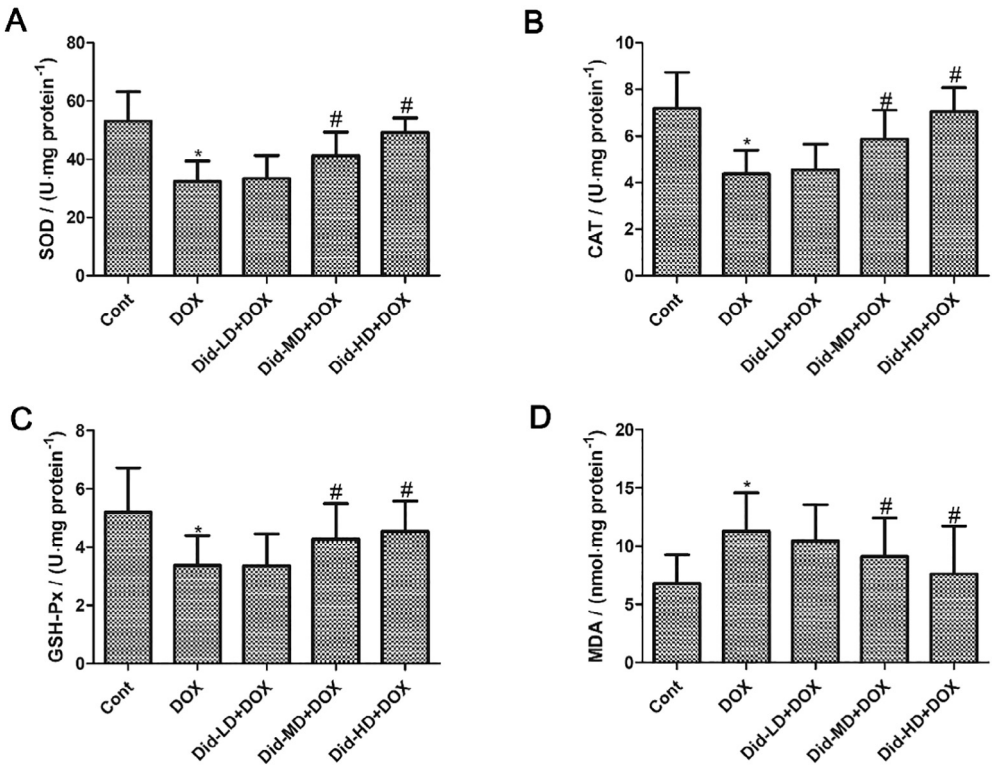

Fig. 4. Effects of Did and DOX on SOD (A), CAT (B), GSH-px (C), and MDA (D) activities in heart tissues of mice (mean ± SD, $n = 10$). *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group. Did-LD + DOX: low-dose Did group (2 mg/kg); Did-MD + DOX: medium-dose Did group (4 mg/kg); Did-HD + DOX: high-dose Did group (8 mg/kg).

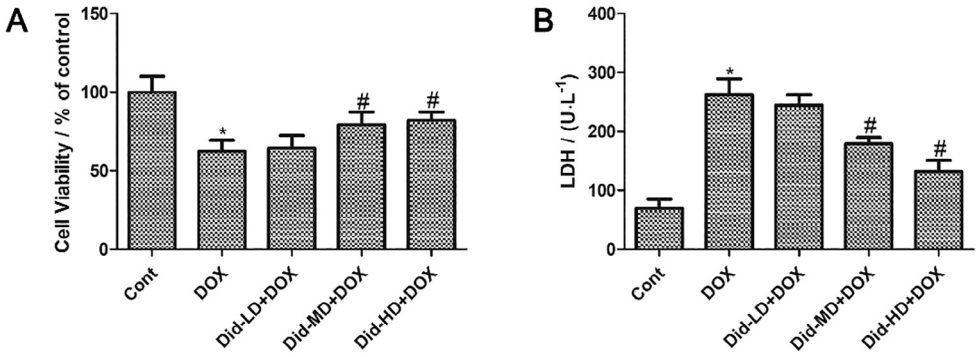

Fig. 5. Effects of Did and DOX on cell viability (A) and LDH release (B) *in vitro*. H9c2 cells were treated with different concentrations of Did for 4 h, and further exposed to 1 μmol/L DOX for 24 h (mean ± SD, $n = 8$).. *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group. Did-LD + DOX: low-dose Did group (5 μg/ml); Did-MD + DOX: medium-dose Did group (10 μg/ml); Did-HD + DOX: high-dose Did group (20 μg/ml).

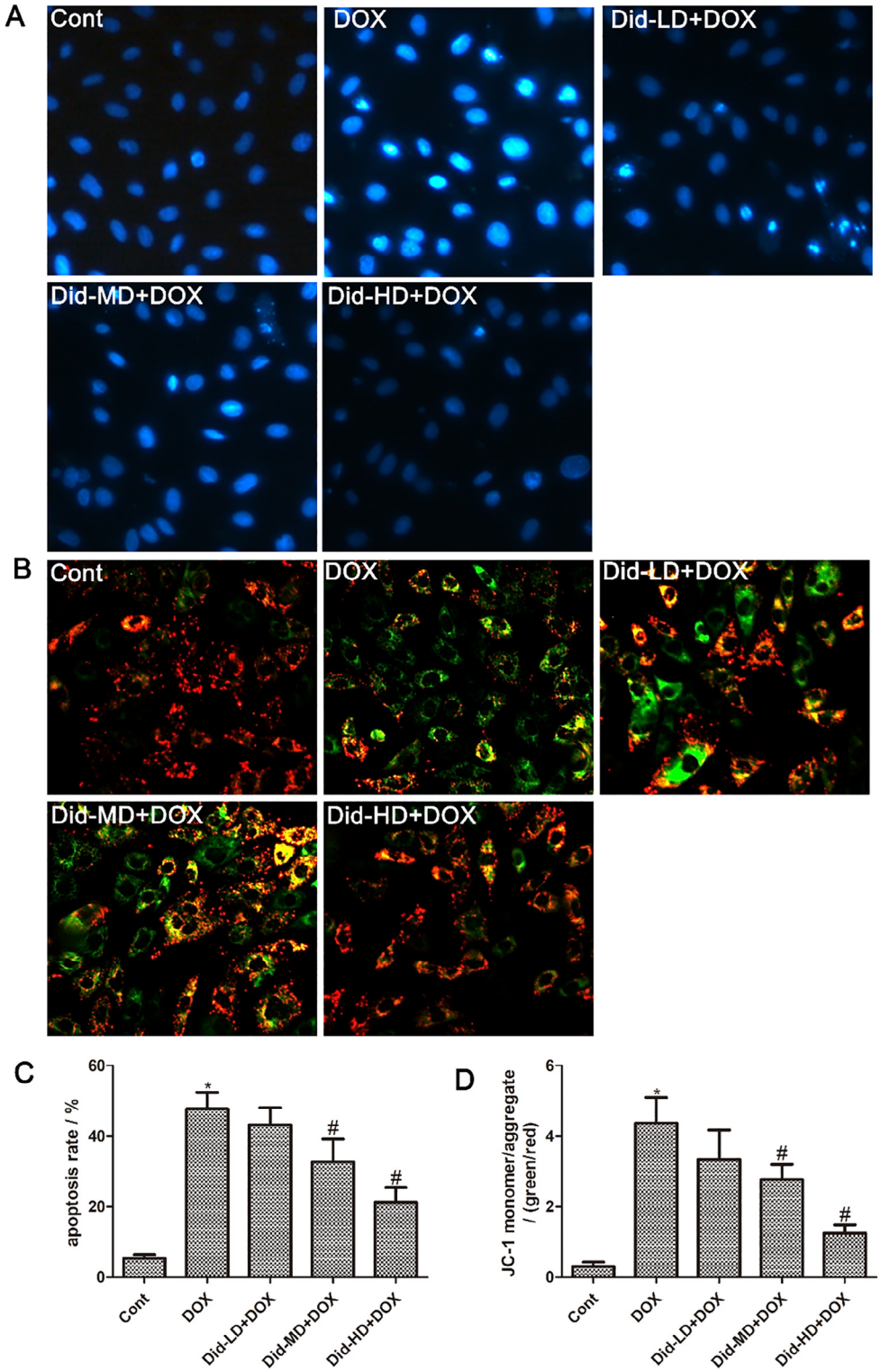

Fig. 6. Effects of Did and DOX on apoptosis *in vitro* (mean ± SD, $n = 8$). (A) Hoechst 33342 staining was used to measure the apoptosis in H9c2 cells. (B) Cells stained with JC-1 dye were visualized by fluorescence microscopy. Quantitative analysis of Hoechst 33342 (C) and JC-1 (D) staining was evaluated. *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group. Did-LD + DOX: low-dose Did group (5 μg/ml); Did-MD + DOX: medium-dose Did group (10 μg/ml); Did-HD + DOX: high-dose Did group (20 μg/ml).

Did pretreatment reduced DOX-induced ROS production significantly. We checked the oxidative stress-associated enzymology index in H9c2 Cells. As shown in Fig. 8, cells treated with DOX showed a significant decrease in the activities of SOD, CAT, GSX-px, GCL, and HO-1 and an increase in the formation of MDA. Pretreatment with Did (5, 10, and 20 μg/ml) before DOX administration reversed these effects. Did could considerably improve cellular antioxidative defense capacity against oxidative stress, which was consistent with the results *in vivo*.

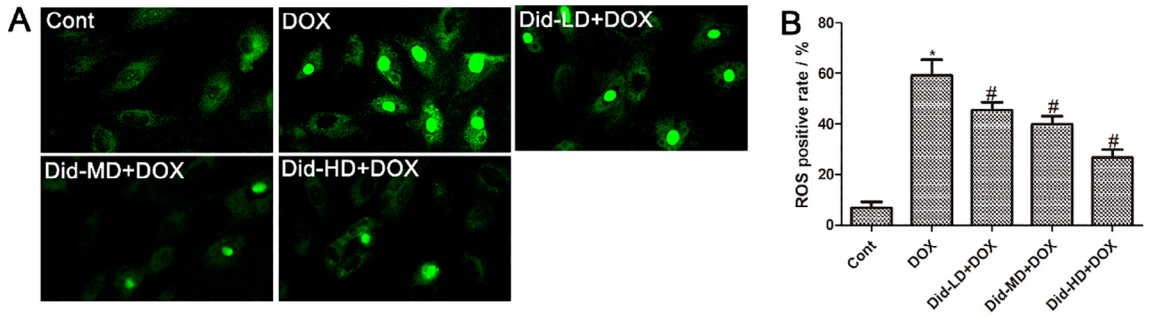

Fig. 7. ROS levels in different groups by fluorescence staining (mean ± SD, $n = 8$). *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group. Did-LD + DOX: low-dose Did group (5 μg/mL); Did-MD + DOX: medium-dose Did group (10 μg/mL); Did-HD + DOX: high-dose Did group (20 μg/mL).

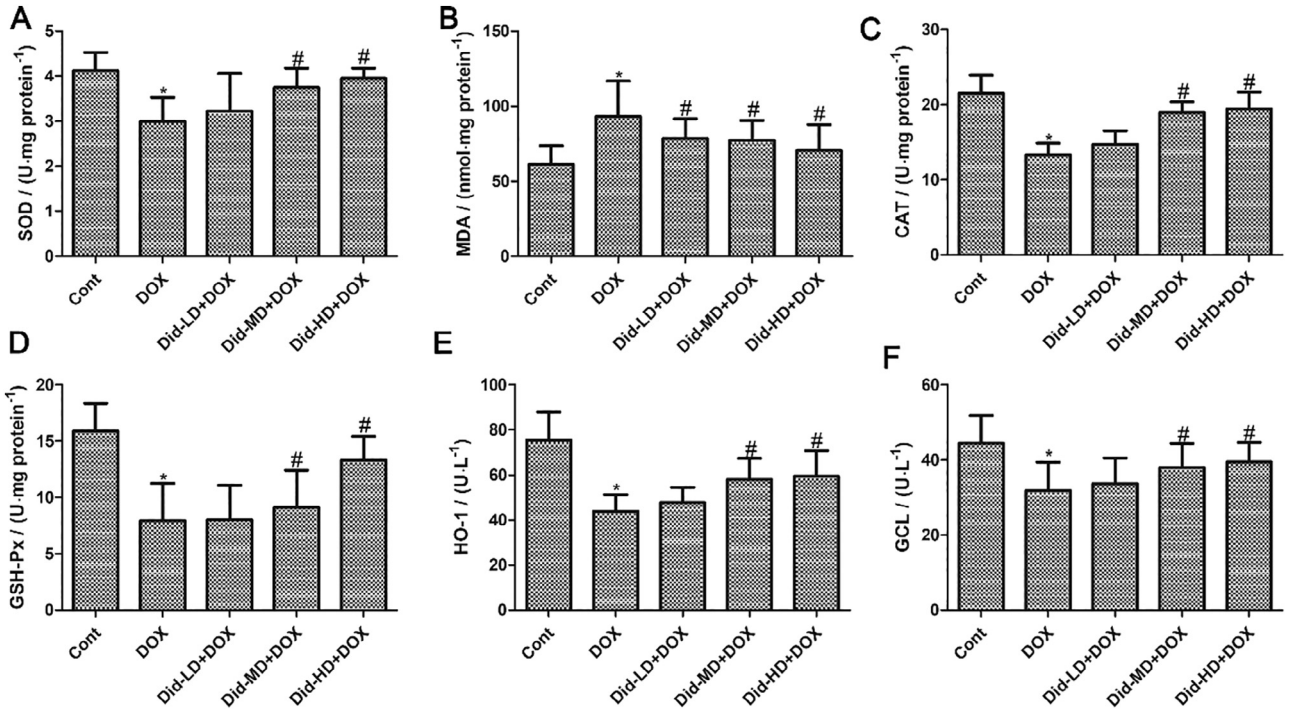

Fig. 8. Effects of Did and DOX on SOD (A), MDA (B), CAT (C), and GSH-Px (D) activities and HO-1 (E), and GCL (F) levels in H9c2 (mean ± SD, $n = 8$). *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group. Did-LD + DOX: low-dose Did group (5 μg/mL); Did-MD + DOX: medium-dose Did group (10 μg/mL); Did-HD + DOX: high-dose Did group (20 μg/mL).

3.6. Did regulated apoptosis related protein expression in H9c2 cells

Did showed protective effect against DOX-induced apoptosis in H9c2 cells. Therefore, we subsequently evaluated the expression levels of Bcl-2 proteins, including proapoptotic Bax and antiapoptotic Bcl-2. The results of Western blot assay showed that DOX elevated Bax protein levels and decreased Bcl-2 protein levels in H9c2 cells, which were mitigated by Did pretreatment. Disruption of mitochondrial membrane permeability results in mitochondrial cytochrome *c* (Cyt-c) release to the cytosol, which will induce cell apoptosis by activating apoptotic effector Caspase 3/9. DOX increased Bax and Caspase 3/9 expression and decreased Bcl-2 expression. The adverse effect of DOX was inhibited by Did treatment (Fig. 9 A and B).

3.7. Effects of Did and DOX on Nrf2 and PI3K/AKT signaling in H9c2 cells

GCL and HO-1 are two well-known target genes of Nrf2. As shown in Fig. 9 C and D, DOX induced a decrease in nuclear Nrf2 expression. In contrast, Did pretreatment significantly increased the protein level of Nrf2 in nuclear. Besides, DOX exposure decreased the protein expression of GCL and HO-1, which was inhibited by Did pretreatment.

Levels of AKT phosphorylation and PI3K expression decreased significantly in DOX group, which was reversed by pretreatment with Did. The results indicated that PI3K/AKT signaling pathway was involved in the cardiac protective effect of Did.

4. Discussion

To the best of our knowledge, the present study is the first to show that Did protects against DOX-induced cardiomyocyte injury and apoptosis, which may be mediated by PI3K/Akt/Nrf2 signaling activation.

CK, AST, and LDH are three cytosolic enzymes that serve as sensitive indices to evaluate the severity of myocardial injury. Increased levels of these enzymes in serum indicate cellular damage, loss of functional integrity, and/or permeability of cell membrane (Chen et al., 2016). DOX administration decreased the body

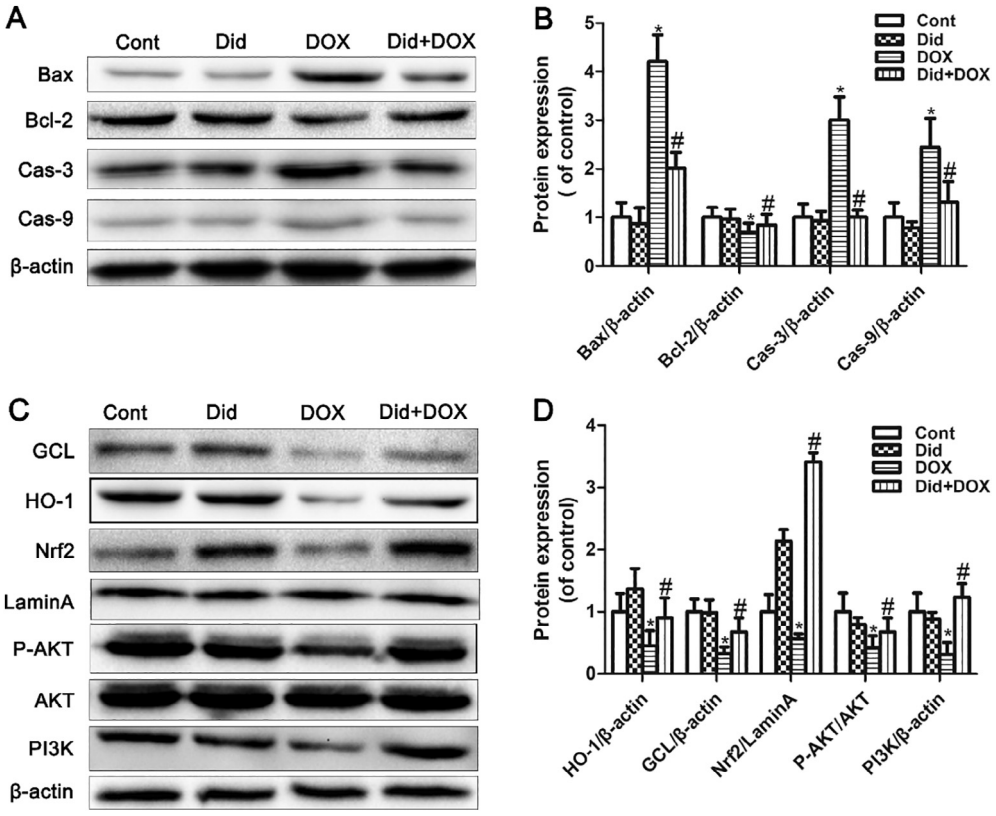

Fig. 9. Protein levels of Bax, Bcl-2, Caspase-3 and Caspase-9 in H9c2 cells determined using (A) Western blot analysis and (B) expressed as the fold changes over the control. The protein levels of phosphorylated and total AKT, PI3K, Nrf2, HO-1, and GCL were measured using (C) Western blot analysis and (D) were expressed as the fold changes over the control. Results are represented as the mean ± SD (n = 8). *P < 0.05 *vs* control group, and #P < 0.05 *vs* DOX group.

weight and increased serum levels of LDH, AST, and CK in mice. DOX administration induced histological damage and decreased EF and FS in the heart. However, Did pretreatment reversed this pathophysiological condition. Experiments *in vitro* also proved that pretreatment with Did effectively increased cell viability and suppressed DOX-induced LDH release in H9c2 cells. Previous studies showed that the toxic effect of DOX on cardiomyocytes is mediated by inducing the generation of oxygen free radical, which provoke cell apoptosis (Catanzaro et al., 2019, Benjanuwattra et al., 2020). We proved that treatment with DOX could increase the level of ROS in cardiomyocytes and depress activities of antioxidant enzymes in heart tissue and cardiomyocytes, indicating that DOX induces apoptosis on myocardial cells by leading to oxygen free radical generation and impeding intrinsic antioxidant capacity. Did pretreatment protected DOX-induced cell injury by enhancing intrinsic antioxidant capacity and inhibiting ROS production.

In order to explore mechanistic pathway, the influence of Did on Nrf2/ARE pathway was examined in this study. HO-1 is an important antioxidant enzyme, which play important function in degrading heme (a potent oxidant) to bilirubin (which has antioxidant properties) (Tomczyk et al., 2019). Study shows that high expression of HO-1 reduces DOX-induced myocardial damage (Koleini et al., 2018). As a thiol-reduced form of glutathione, GSH plays a key role in detoxicating xenobiotics and/or their metabolites. GCL is important in promoting GSH biosynthesis (Guerreiro, Abreu, Fillmann, & Sandrini, 2020). All these enzymes are regulated by Nrf2. In response to oxidative stress, activated Nrf2 acts as key transcriptional factor that triggers ARE pathway and leads to transcriptional activation of antioxidant enzymes, including HO-1 and GCL (Zhao et al., 2018). DOX-administration decreased the expression of HO-1 and GCL in the present study. Pretreatment with Did increased the localization of Nrf2 in nucleus, and enhanced the expression of HO-1 and GCL. Did probably modulated the interactions between Keap1 and Nrf2, which activated nuclear translocation of Nrf2, thereby promoting the expressions of HO-1 and GCL. Moreover, the enhancement in the activities of other antioxidant enzymes, such as SOD, CAT, and GSH-Px, validated the involvement of Nrf2-mediated activation of ARE pathway in Did-mediated cardio protection.

PI3K/Akt signaling pathway is an important cell survival signal involved in many pathophysiological (Hoxhaj and Manning, 2020). Our previous study suggested that AKT activation could prevent DOX-induced cardiomyocytes apoptosis and improve contractile function of heart (Chen et al., 2015). PI3K/AKT is also involved in Nrf2 activation (Fu et al., 2020). In the present study, DOX inhibited the expression of PI3K and p-AKT in cardiomyocytes, and such effect was attenuated by Did pretreatment. These results suggested that PI3K/AKT signaling pathway may participate in protective effect of Did on DOX-induced cardiotoxicity.

5. Conclusion

The present study proved that Did inhibited DOX-induced cardiotoxicity *in vivo* and *in vitro* by alleviating oxidative stress injury. The protective effects were partially attributed to the activation of the PI3K/Akt/Nrf2 pathway. These results serve as reference for the development of Did into cardiovascular drugs. However, advanced research is necessary to further explore the mechanism of Did against DOX-induced myocardial damage.

Editor Note

Xiaobo Sun is Editorial Board Members of Chinese Herbal Medicines. He was blinded from reviewing or making decisions on the manuscript. The article was subject to the journal's standard procedures, with peer review handled independently of this Editorial Board Member and their research groups.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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