

Original Article

α -Linolenic acid attenuates doxorubicin-induced cardiotoxicity in rats through suppression of oxidative stress and apoptosis

Xiaohua Yu¹, Libao Cui², Zizhen Zhang³, Qihui Zhao³, and Shuangjie Li^{4*}

¹Life Science Research Center, University of South China, Hengyang 421001, China

²Department of Pharmacy, Central Hospital of Hengyang City, Hengyang 421000, China

³School of Nursing, Hunan Polytechnic of Environment and Biology, Hengyang 421001, China

⁴Center of Liver Disease Research, Hunan Children's Hospital, Changsha 410007, China

*Correspondence address. Tel/Fax: +86-731-85356922; E-mail: lesjie62@vip.sina.com

Doxorubicin (DOX), a widely used anti-tumor drug, can give rise to severe cardiotoxicity by oxidative stress and cell apoptosis, which restricts its clinical application. α -Linolenic acid (ALA) has been shown to serve as a potent cardioprotective agent. The aim of this study was to explore the protective effects of ALA on DOX-induced cardiotoxicity and the underlying molecular mechanisms for this cardioprotection in rats. Rats were randomly divided into four groups and administrated with normal saline, ALA (500 μ g/kg), DOX (2.5 mg/kg), or ALA (500 μ g/kg) plus DOX (2.5 mg/kg) for 17 days. The results showed that DOX treatment significantly increased the heart weight/body weight, liver wet weight (WW)/dry weight (DW), lung WW/DW, serum levels of brain natriuretic peptide, creatine kinase-MB, lactate dehydrogenase, and cardiac troponin I, myocardial necrosis and myocardial malondialdehyde content, and induced the mRNA expression of *Nrf2* in the nucleus, cleaved caspase-3, *Bax*, and superoxide dismutase (*SOD*). In addition, DOX led to a significant decrease in left ventricular end-diastolic volume, stroke volume, ejection fraction, SOD, glutathione-peroxidase, catalase, as well as the expression of Kelch-like ECH-associated protein 1 (*Keap1*) in the cytoplasm, phospho-AKT, phospho-ERK, and *Bcl-2*. Co-treatment with ALA significantly eliminated these changes induced by DOX except further reduction of *Keap1* and elevation of *Nrf2* and *SOD* mRNA. These results showed the cardioprotective effects of ALA on DOX-induced cardiotoxicity in rats. The mechanisms might be associated with the enhancement of antioxidant defense system through activating *Keap1/Nrf2* pathway and anti-apoptosis through activating protein kinase B/extracellular signal regulated kinase pathway. Our results suggested a promising future of ALA-based preventions and therapies for myocardial damage after administration of DOX.

Keywords doxorubicin; α -linolenic acid; oxidative stress; apoptosis

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Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is widely used in the treatment of a variety of solid and hematological malignancies. However, the clinical application of this chemotherapeutic drug is restricted by its severely cumulative dose-dependent cardiotoxicity that can cause irreversible heart failure [1]. The exact pathogenesis of DOX-induced cardiotoxicity is not fully understood, but many different mechanisms have been provided, including oxidative stress, intracellular calcium overload, myofibrillar degeneration, cytokine release, and induction of cardiomyocyte apoptosis [2]. Among these mechanisms, oxidative stress and cardiomyocyte apoptosis are the most likely activators [3]. Accordingly, a number of studies have been performed with the aim to inhibit DOX-induced oxidative insult and cell apoptosis to antagonize its cardiotoxicity [4,5], although, to date, no single agent has been proven clinically effective and safe in the prevention of this serious side effect of DOX. Thus, it is of utmost importance to develop novel therapeutic approaches to prevent the cardiotoxicity of DOX.

Bordoni *et al.* [6] recognized the impairment of essential fatty acid metabolism as a critical factor in DOX-induced damage in cultured rat cardiomyocytes. They observed that DOX has a dual negative effect, depending on its concentration and the time period of exposure. That is, one directed against the membrane highly unsaturated fatty acids (HUFAs), the other against the system that is required for the synthesis of these fatty acids themselves from their precursors, linoleic acid and α -linolenic acid (ALA). Inhibition of

these effects might be useful to counteract DOX toxicity, so the administration of exogenous HUFAs could be critical in filling the reduced unsaturated fatty acid pool. The possibility of counteracting DOX cardiotoxicity by administration of antioxidants and γ -linolenic acid (GLA) was proposed [6]. Then, direct evidence showed that HUFA biosynthesis plays a role in counteracting DOX toxicity but it cannot completely overcome the depletion of these fatty acids, and that serum and exogenous GLA are critical in filling the decreased HUFA pool [7]. In another study, green tea extracts have been reported to maintain the unsaturation index similar to that observed in cardiomyocytes without exposing to DOX by preventing modifications of HUFAs, thereby contributing to protection against cardiotoxicity of DOX [8]. In addition, the *in vitro* and *in vivo* anti-tumor activity studies indicated that the DOX-ALA conjugate is more cytotoxic than free DOX [9,10]. However, the role of ALA in DOX-induced cardiotoxicity still remains elusive.

ALA is one of the precursors of HUFAs and its cardioprotective effects have been investigated. It has been shown that higher ALA exposure is associated with a moderately lower risk of cardiovascular diseases [11]. *In vivo* studies have demonstrated that dietary supplement of the plant-derived ALA for 4 weeks significantly alleviated myocardial ischemia/reperfusion injury in rats through suppression of oxidative stress due to decreased free radical and lipid peroxidation production [12]. Xie *et al.* [13] also found that ALA intake confers cardioprotection in myocardial ischemia/reperfusion by exerting anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. In addition to these anti-oxidative cardioprotective effects, ALA also has anti-apoptotic properties. For instance, pretreatment with ALA has been reported to protect against myocardial cell apoptosis by inhibition of reactive oxygen species (ROS) generation, thereby contributing to reducing myocardial infarction size [14]. Taken together, these findings provided evidence that ALA may have potential therapeutic value for cardiac damage induced by oxidative stress and pro-apoptotic effects.

Since the critical mechanisms of DOX-induced cardiotoxicity are associated with oxidative stress and cell apoptosis, we hypothesized that ALA might also ameliorate the adverse effects of DOX on cardiac tissues. To test this possibility, in the current study, we explored the protective effects of ALA against cardiotoxicity induced by DOX in rats and the underlying molecular mechanism. We demonstrated that ALA significantly protects myocardium from DOX-mediated cardiotoxicity by anti-oxidative stress via activating Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, and by anti-apoptosis via activating protein kinase B (AKT)/extracellular signal regulated kinase (ERK) pathway. Our results may provide a rational basis for this fatty acid to be an antidote to cardiac toxicity induced by DOX in clinical practice.

Materials and Methods

Experimental animals

The experiments were approved by the Animal Care and Use Committee of University of South China (Hengyang, China) and were performed in compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male Sprague-Dawley rats, weighing 220 ± 20 g, were obtained from the Animal Department, University of South China. The rats were housed in metabolic cages under controlled environmental conditions (25°C and a 12/12 h light/dark cycle) with free access to food and water.

Drugs and chemicals

ALA with a purity of 95% was a gift from the Medical College, Jishou University (Jishou, China). DOX was purchased from Zhejiang Hisun Pharmaceutical (Taizhou, China). The primary antibodies against Bcl-2, cleaved caspase-3, AKT, phospho-AKT (p-AKT), ERK, and phospho-ERK (p-ERK) were obtained from Cell Signaling Technology (Beverly, USA). Antibodies against Keap1, Nrf2, and Bax were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). ReverAid™ First-Strand cDNA Synthesis Kit (#k1622) (Fermentas, Burlington, Canada), DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland), BCA Protein Assay Reagent (Pierce Chemical, Rockford, USA), and nitrocellulose membranes (Millipore, Boston, USA) were obtained as indicated. All other biochemical reagents and chemicals were of analytical grade.

Experimental protocol

After acclimatization, the animals were randomly divided into four groups each with 10 rats: control group, ALA group, DOX group, and DOX plus ALA group. Rats in ALA group and DOX plus ALA group were orally administered daily with ALA (500 μ g/kg) dissolved in 0.5 ml of 0.01% alcohol by gastric gavage for 3 days, whereas rats in the other two groups received an equal volume of normal saline. From the beginning of the fourth day, the animals were treated every alternate day for seven times (total period, 14 days) as follows: rats in control group were intraperitoneally injected with normal saline; rats in DOX group were intraperitoneally inoculated with 2.5 mg/kg of DOX; rats in DOX plus ALA group were administered with 500 μ g/kg of ALA through gastric gavage 1 h before injection with 2.5 mg/kg of DOX; rats in ALA group were treated with 500 μ g/kg of ALA through gastric gavage. Body weight (BW), mortality, and general appearance were observed and recorded daily.

After 24 h of last dose of DOX, the rats were anesthetized by pentobarbital sodium, and echocardiography measurement was performed. Blood samples were obtained from celiac

aorta, and serum was subsequently separated for detection of brain natriuretic peptide (BNP), cardiac troponin I (cTnI), creatine kinase-MB (CK-MB), and lactate dehydrogenase (LDH). After blood collection, the rats were executed by cervical decapitation. The livers and lungs were excised, gently cleaned with gauze, weighed for wet weight (WW), and dried at 37°C in a warm incubator for ~72 h. Then, these livers and lungs were reweighed for dry weight (DW) until their weight was constant. At the same time, the hearts were quickly removed, rinsed in ice-cold normal saline, and weighed as heart weight (HW) for analysis of diverse biochemical parameters.

Detection of serum BNP, cTnI, CK-MB, and LDH levels

The levels of BNP and cTnI in serum were measured by enzyme-linked immunosorbent assay using standard kits (Nanjing Jiancheng Bio-Engineering Institute, Nanjing, China) according to the manufacturer's instruction. CK-MB and LDH activities in serum were detected using commercially available assay kits (Biosino Bio-Technology and Science Inc., Beijing, China).

Histopathological examination

Samples of cardiac tissue were fixed in 10% acetate-buffered formalin, and then embedded in paraffin. The paraffin blocks were sectioned at 5 µm and stained with hematoxylin and eosin (H&E). A pathologist blinded to the treatments carried out the histopathological examination under an optical microscopy (Olympus, Tokyo, Japan).

Echocardiography

After anesthetization with pentobarbital sodium, the rats were imaged in the supine position using a 17.5-MHz center frequency RMV 707 scanhead. 2D B-mode and M-mode images were acquired by a technician who was blinded to the experimental protocol. Left ventricular (LV) end-diastolic and end-systolic diameters were examined on the M-mode parasternal short-axis tracing at papillary muscle level. Left ventricular end-diastolic volume (LVEDV), stroke volume (SV), and LV ejection fraction (EF) were obtained by software analysis. The data were averaged from at least three consecutive cardiac cycles.

Measurement of lipid peroxidation products and antioxidants

Cardiac tissue samples were homogenized in 10 ml of phosphate buffer at 4°C with a homogenizer (IKA, Staufen, Germany). Then, the homogenate was centrifuged at 352 g for 30 min and the supernatant was collected. The protein content was determined by BCA protein assay reagent. Measurement of lipid peroxidation by determination of myocardial malondialdehyde (MDA) content was conducted using MDA assay kit (Nanjing Jiancheng Bio-engineering Institute). Antioxidant enzymes namely glutathione-peroxidase

(GSH-Px), superoxide dismutase (SOD), and catalase (CAT) were evaluated with commercially available assay kits (Nanjing Jiancheng Bio-Engineering Institute).

Real-time quantitative polymerase chain reaction analysis

Total RNA was extracted from cells using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). The first-strand cDNA was synthesized using ReverAid™ First-Strand cDNA Synthesis Kit according to the manufacturer's protocol. Total RNA (1 µg) and oligo (dT) were used. Real-time quantitative polymerase chain reaction (PCR) using SYBR Green detection chemistry was performed on Roche Light Cycler Run 5.32 Real-Time PCR System (Roche, Basel, Switzerland). Specific primers for *SOD* and *GAPDH* were designed as follows: *SOD* forward primer, 5'-CCTTCTTGTCTGC AACCTGCTA-3' and reverse primer, 5'-CCGGACTCTCC GGTATCTGA-3'; *GAPDH* forward primer, 5'-CAGCCT CGTTCATAGACAAGATG-3' and reverse primer, 5'-CA ATGTCCAC TTTGTCACAAGAGAA-3'. Melt curve analyses of all real-time PCR products were performed and shown to produce a single DNA duplex. Quantitative measurements were determined using the $\Delta\Delta C_t$ method and the expression of *GAPDH* was used as the internal control.

Determination of myocardial apoptosis

Myocardial apoptosis was determined using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay as previously described [15]. The fragmented DNA on the 5-µm tissue sections was labeled with the ApopTag apoptosis detection kit (Roche). In brief, after proteinase K digestion and quenching of endogenous peroxidase using 0.3% H₂O₂ solution, formalin-fixed paraffin slides were incubated with the reaction mixture that included working solution of TdT and digoxigenin-conjugated dUTP for 1 h at 37°C. Then, labeled DNA was measured through peroxidase-conjugated anti-digoxigenin conjugate antibody. The bound complex was stained with a 3,3'-diaminobenzidine tetrahydrochloride-based substrate. The apoptotic index was expressed as the percentage of apoptotic cell number to the total number of myocardial cells. The assays were performed in a blinded fashion.

Western blot analysis

The cytosolic and nuclear proteins were extracted as described previously [16,17]. Supernatants were used as sample proteins. Protein concentrations were determined using BCA protein assay reagent. Equal amounts of proteins (20 µg) were applied to 10% sodium dodecyl sulfate gels and then transferred to nitrocellulose membranes after electrophoresis. The membranes were incubated with various primary antibodies at a dilution of 1 : 1000 at 4°C overnight.

After being washed, the membranes were incubated with anti-rabbit IgG labeled with horseradish peroxidase (Santa Cruz) diluted at 1 : 1000 at room temperature for 2 h. The proteins were visualized using a chemiluminescence method (ECL Plus Western Blotting Detection System; Amersham Biosciences, Foster City, USA). Densitometry was carried out by Image J software (version 1.38).

Statistical analysis

Data are expressed as the mean \pm SD. The significance of inter-group differences was evaluated by one-way analyses of variance (least-significant difference's test for *post hoc* comparisons), using SPSS 13.0 software. Differences were considered statistically significant at $P < 0.05$.

Results

ALA ameliorates cardiac function in DOX-treated rats

At the end of the treatment period, all rats were alive. The rats in control and ALA groups were normal while those in DOX and ALA + DOX groups appeared weak. Echocardiographic examination demonstrated that DOX treatment significantly reduced LVEDV, SV, and EF in rats. In contrast, LVEDV, SV, and EF were higher in ALA + DOX group than those in DOX group (Table 1). In addition, serum BNP levels, HW/BW, liver WW/DW, and lung WW/DW were markedly increased in DOX group compared with control group, whereas the increase was significantly inhibited by ALA pretreatment (Table 2). Of note, ALA alone

had no impacts on these indicators. Thus, these data suggested that ALA contributes to the improvement of cardiac function after DOX administration in rats.

ALA prevents DOX-induced elevation of serum CK-MB, LDH, and cTnI levels

The intracellular CK-MB, LDH, and cTnI are released into the blood stream after disruption of cardiomyocytes. Thus, CK-MB, LDH, and cTnI in serum are recognized as excellent markers for cardiac injury [18]. As shown in Table 3, compared with serum levels of CK-MB, LDH, and cTnI in control group, treatment with ALA alone did not affect them. These three markers were significantly higher in DOX group than those in normal group. However, pretreatment with ALA in DOX-administered rats showed a significant reduction at the levels of all serum markers compared with DOX-administered rats, further confirming the cardioprotective effects of ALA on DOX-induced myocardial injury in rats.

ALA improves DOX-induced cardiac histopathological changes

To further confirm the results obtained from these biochemical assays, histopathological examination of hearts was performed using H&E staining. The cardiac tissues from control and DOX alone-treated rats showed a normal myofibrillar structure with striations, branched appearance, and continuity with adjacent myofibrils. Extensive

Table 1 Effects of ALA on cardiac pump function

Group	<i>n</i>	LVEDV (μ l)	SV (μ l)	EF (%)
Control	10	207.65 \pm 18.24	145.27 \pm 16.38	72.32 \pm 6.54
ALA	10	213.19 \pm 20.68	152.19 \pm 17.23	74.31 \pm 6.95
DOX	10	148.42 \pm 10.76*	90.61 \pm 8.64*	48.25 \pm 4.13*
ALA + DOX	10	186.92 \pm 16.17 ^{&}	128.76 \pm 11.07 ^{&}	64.91 \pm 5.63 ^{&}

LVEDV, left ventricular end-diastolic volume; SV, stroke volume; EF, ejection fraction.

* $P < 0.05$ vs. control group.

[&] $P < 0.05$ vs. DOX group.

Table 2 Effects of ALA on serum BNP levels, HW/BW, liver WW/DW, and lung WW/DW

Group	<i>n</i>	BNP (ng/ml)	HW/BW (mg/g)	Liver WW/DW	Lung WW/DW
Control	10	76.78 \pm 4.29	3.02 \pm 0.21	3.18 \pm 0.25	4.78 \pm 0.36
ALA	10	73.12 \pm 4.51	3.11 \pm 0.23	3.24 \pm 0.21	4.71 \pm 0.32
DOX	10	125.02 \pm 6.27*	4.47 \pm 0.32*	4.29 \pm 0.36*	6.87 \pm 0.54*
ALA + DOX	10	90.21 \pm 5.24 ^{&}	3.52 \pm 0.28 ^{&}	3.32 \pm 0.28 ^{&}	5.02 \pm 0.43 ^{&}

BNP, brain natriuretic peptide; BW, body weight; HW, heart weight; WW, wet weight; DW, dry weight.

* $P < 0.05$ vs. control group.

[&] $P < 0.05$ vs. DOX group.

Table 3 Effects of ALA on serum levels of CK-MB, LDH, and cTnI

Group	n	CK-MB (IU/l)	LDH (IU/l)	cTnI (ng/ml)
Control	10	68.71 ± 3.42	54.16 ± 4.62	0.26 ± 0.04
ALA	10	74.18 ± 3.63	59.12 ± 5.13	0.31 ± 0.05
DOX	10	201.53 ± 9.67**	148.69 ± 7.94**	1.14 ± 0.16**
ALA + DOX	10	134.49 ± 8.78&&	79.37 ± 6.04&&	0.64 ± 0.09&&

CK-MB, creatine kinase-MB; LDH, lactate dehydrogenase; cTnI, cardiac troponin I.

** $P < 0.01$ vs. control group.

&& $P < 0.01$ vs. DOX group.

cardiomyocyte necrosis was found in DOX group. However, DOX-caused histopathological change was significantly attenuated in ALA + DOX group (Fig. 1).

ALA blocks DOX-induced changes of lipid peroxidation and antioxidant enzymes

MDA is a naturally occurring product of lipid peroxidation, and its concentration is directly proportional to the tissue damage caused by oxidative stress [19]. SOD, GSH-Px, and CAT are three critical enzymes in the detoxification of ROS, which have been shown to be protective against various forms of oxidative myocardial damage [20,21]. We, therefore, measured the levels of MDA, SOD, GSH-Px, and CAT in cardiac tissues. As shown in Table 4, DOX alone significantly raised myocardial MDA level but decreased myocardial SOD, GSH-Px, and CAT levels compared with those in control group, whereas co-treatment with ALA and DOX could effectively prevent DOX-induced changes. In addition, rats treated with DOX markedly increased the expression of *SOD* mRNA in myocardium, and the increase was promoted by ALA (Fig. 2). No significant difference was observed between ALA group and control group with regard to these indicators. These results supported the idea that ALA can attenuate cardiotoxicity of DOX due to its anti-oxidative properties.

Keap1/Nrf2 pathway is involved in anti-oxidative effect of ALA

Nrf2, a nuclear transcription factor, is regarded as the most important factor that protects cells or tissues against oxidative stress generated from exposure to exogenous and endogenous chemicals, metals, and radiation [22]. Under unstimulated conditions, Nrf2 is retained in the cytoplasm by forming a covalent complex with an inhibitor Keap1. The presence of a stimulus results in the disruption of the Keap1–Nrf2 complex and nuclear translocation of Nrf2, thereby inducing gene transcription of a majority of antioxidant enzyme genes such as *SOD*, *GSH-Px*, and *CAT* [23,24]. To explore whether Keap1/Nrf2 signaling pathway was involved in ALA's anti-oxidative effect, we detected the impacts of ALA and/or DOX treatment on the expression of

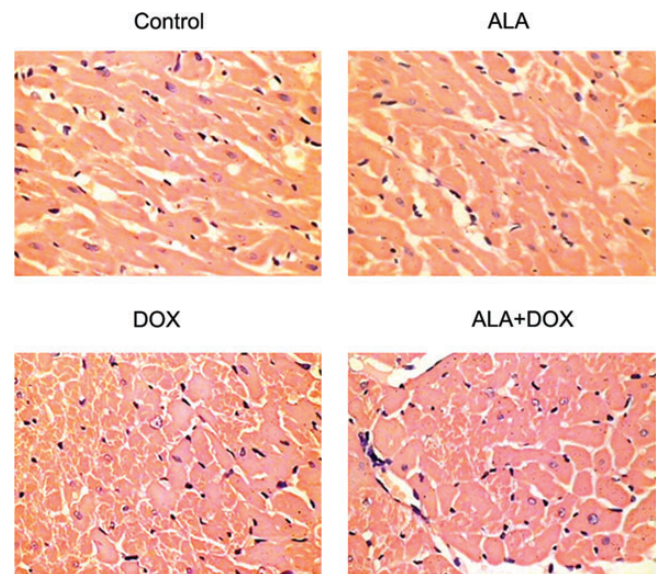


Figure 1 Representative photomicrographs of rat myocardium The myocardial sections were stained with H&E (magnification, $\times 400$). Control, control group; ALA, α -linolenic acid group; DOX, doxorubicin group; ALA + DOX group, α -linolenic acid plus doxorubicin group.

Keap1 in the cytoplasm and Nrf2 in the nucleus using western blot analysis. As expected, treatment with DOX alone significantly reduced cytosolic Keap1 level but increased nuclear Nrf2 level compared with control group, and these changes were further promoted in response to ALA and DOX (Fig. 3). In contrast, there was no statistical significance at the Keap1 and Nrf2 levels between ALA group and control group. These data strongly supported the hypothesis that DOX administration leads to the degradation of Keap1 and thus facilitates Nrf2 nuclear translocation, and that ALA further promotes these actions.

ALA suppresses DOX-induced cardiomyocyte apoptosis

Cardiomyocyte apoptosis plays an important role in the pathogenesis of DOX-induced myocardial injury. To explore the effect of ALA pretreatment on apoptosis induced by DOX in cardiac myocytes, TUNEL staining was used to detect and quantify myocardial apoptosis in all experimental rat hearts, based on the labeling of DNA strand breaks. As

Table 4 Effects of ALA on cardiac MDA, SOD, GSH-Px, and CAT levels

Group	<i>n</i>	MDA (nmol/mg protein)	SOD (U/mg protein)	GSH-Px (U/mg protein)	CAT (U/mg protein)
Control	10	76.82 ± 6.37	68.02 ± 6.15	18.27 ± 2.12	38.12 ± 4.91
ALA	10	72.51 ± 6.12	65.84 ± 6.38	19.46 ± 2.35	40.24 ± 4.62
DOX	10	103.42 ± 8.16**	47.49 ± 3.45**	10.59 ± 1.68**	19.57 ± 3.44**
ALA + DOX	10	84.29 ± 7.25&&	59.82 ± 4.94&&	16.87 ± 2.45&&	32.2 ± 4.01&&

MDA, malondialdehyde; GSH-Px, glutathione-peroxidase; SOD, superoxide dismutase; CAT, catalase.

***P* < 0.01 vs. control group.

&&*P* < 0.01 vs. DOX group.

shown in **Fig. 4**, TUNEL-positive cells were scanty in control group and ALA group. However, abundant TUNEL-positive cells were found in DOX group, and this effect was significantly but not completely suppressed by co-treatment with ALA and DOX. Quantitative analysis demonstrated that the apoptotic index is significantly increased in DOX group compared with control group, and this increase is obviously blocked by pretreatment of ALA. ALA alone has no influence on cardiomyocyte apoptosis. These findings indicated that ALA protects cardiomyocytes from DOX-caused cytotoxicity.

ALA inhibits DOX-induced caspase-3 activation and reverses DOX-induced changes of Bcl-2 and Bax expression

Caspase-3 is a critical executioner of apoptosis. As shown in **Fig. 5(A)**, treatment with DOX significantly elevated the level of cleaved caspase-3 in myocardium, which was markedly inhibited by pretreatment of ALA, suggesting that DOX activates caspase-3 and ALA can suppress DOX-stimulated activation of caspase-3.

Bcl-2 family proteins are localized in mitochondria to modulate the permeability of mitochondrial outer membrane and control downstream mitochondrion-dependent cellular apoptosis [25]. To determine the effects of DOX on anti-apoptotic Bcl-2 and pro-apoptotic Bax expression and the response of ALA to the effects of DOX, the levels of Bcl-2 and Bax were analyzed by western blot analysis. As shown in **Fig. 5(B,C)**, treatment with DOX significantly decreased the amount of Bcl-2 expression but increased the Bax expression. However, co-treatment with ALA significantly abolished DOX-induced decrease of Bcl-2 expression and increase of Bax expression. These results indicated that ALA is able to block the DOX-mediated down-regulation of Bcl-2 expression and up-regulation of Bax expression.

ALA reverses DOX-induced decreases of p-AKT and p-ERK levels

Phosphorylation of AKT and ERK has been suggested to play critical roles in preventing DOX-caused cardiomyocyte

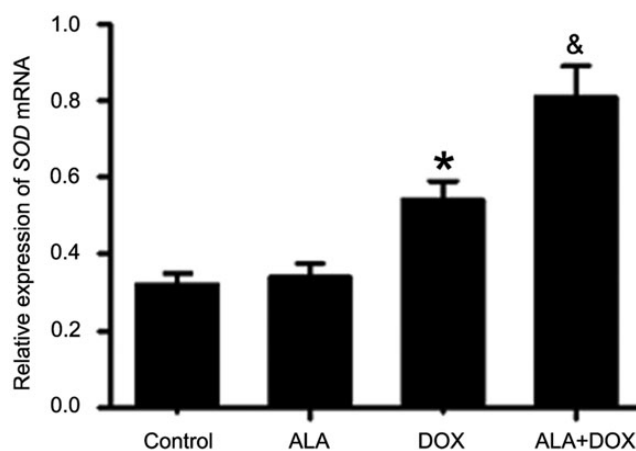


Figure 2 Effects of ALA on DOX-induced *SOD* mRNA expression in rat myocardium. Total RNA was extracted from the cardiac tissues, and real-time quantitative PCR was performed to determine the mRNA expression of *SOD*. Values are expressed as the mean ± SD (*n* = 10). Control, control group; ALA, α-linolenic acid group; DOX, doxorubicin group; ALA + DOX group, α-linolenic acid plus doxorubicin group. **P* < 0.05 vs. control group. &*P* < 0.05 vs. DOX group.

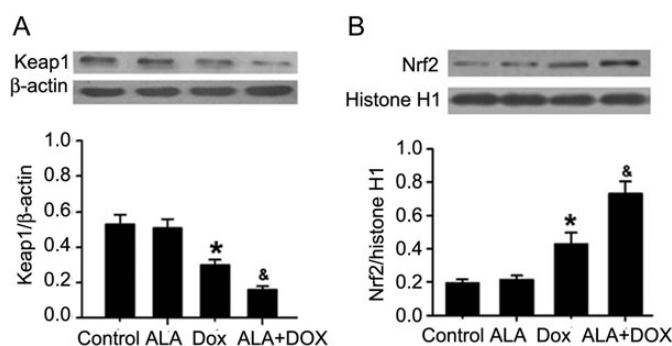


Figure 3 Effects of ALA on DOX-induced changes of Keap1 and Nrf2 expression in rat myocardium. (A) The expression of Keap1 in the cytoplasm was evaluated via western blot analysis using the anti-Keap1 and anti-β-actin antibodies. β-Actin was used as a loading control. (B) The levels of Nrf2 expression in the nucleus were analyzed by western blot analysis using the anti-Nrf2 and anti-histone H1 antibodies. Histone H1 was used as a loading control. Values are expressed as the mean ± SD (*n* = 10). Control, control group; ALA, α-linolenic acid group; DOX, doxorubicin group; ALA + DOX group, α-linolenic acid plus doxorubicin group. **P* < 0.05 vs. control group. &*P* < 0.05 vs. DOX group.

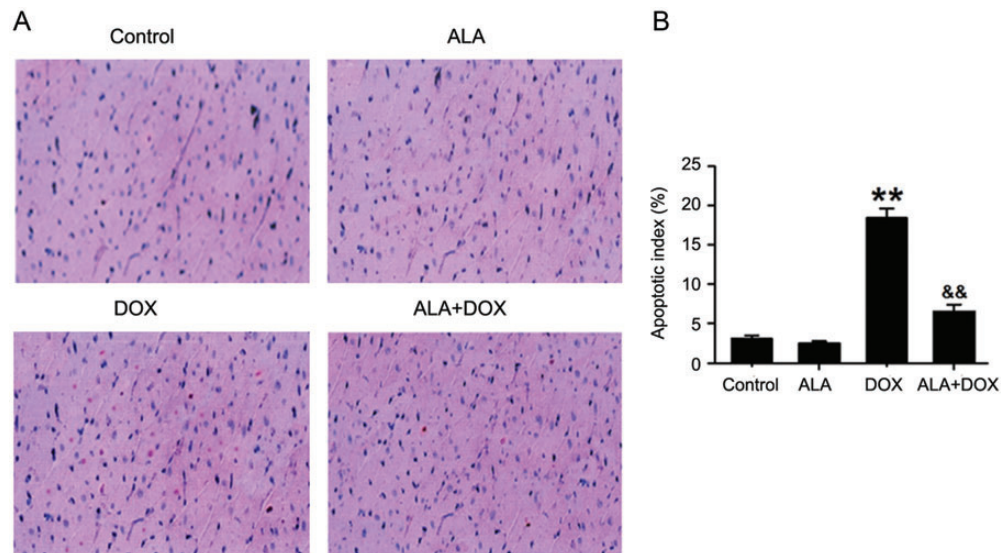


Figure 4 Effects of ALA on DOX-induced cardiomyocyte apoptosis in rats (A) Representative images of TUNEL assay in the sections of cardiac tissue (original magnification, $\times 400$). (B) The apoptotic index was expressed as the ratio of TUNEL-positive cardiomyocyte nuclei to total number of cardiomyocyte nuclei. Data were represented as the mean \pm SD ($n = 10$). Control, control group; ALA, α -linolenic acid group; DOX, doxorubicin group; ALA + DOX group, α -linolenic acid plus doxorubicin group. ** $P < 0.01$ vs. control group. && $P < 0.01$ vs. DOX group.

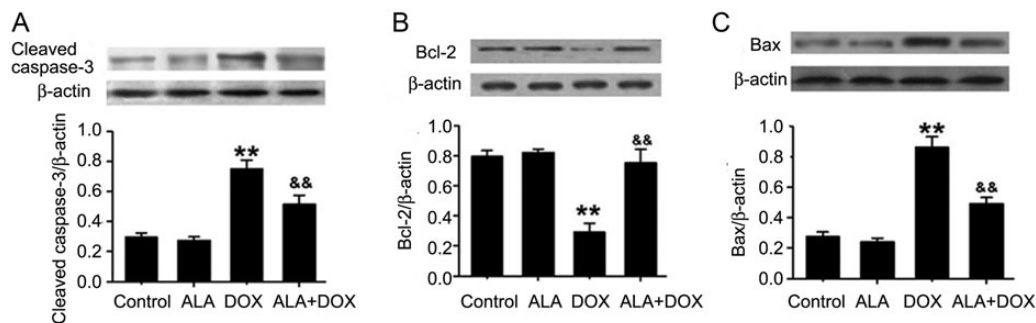


Figure 5 Effects of ALA on caspase-3 activation and changes of Bcl-2 and Bax expression induced by DOX in cardiac tissues (A) The activation of caspase-3 was measured through western blot analysis using the anti-cleaved caspase-3 antibody. β -Actin was used as a loading control. (B,C) The levels of Bcl-2 and Bax expression in cardiac tissues were evaluated by western blot analysis using the anti-Bcl-2 and anti-Bax antibodies. β -Actin was used as a loading control. Values are expressed as the mean \pm SD ($n = 10$). Control, control group; ALA, α -linolenic acid group; DOX, doxorubicin group; ALA + DOX group, α -linolenic acid plus doxorubicin group. ** $P < 0.01$ vs. control group. && $P < 0.01$ vs. DOX group.

apoptosis [26]. To clarify whether this pathway was involved in ALA's cardioprotection, we measured the levels of p-AKT and p-ERK in myocardium using western blot analysis. As expected, the expression levels of p-AKT and p-ERK were significantly decreased in DOX group but were significantly increased by ALA pretreatment (Fig. 6). There was no difference in total AKT and ERK protein expression among all groups tested. These data demonstrated that the anti-apoptotic effect of ALA is associated with activation of AKT/ERK signaling pathway.

Discussion

ALA is one of the two essential fatty acids in humans that must be provided in the diet because the body needs them

but cannot synthesize them. Both epidemiological data and dietary trials strongly supported the fact that ALA has cardioprotective functions [12–14]. Nevertheless, the effects of ALA on DOX-induced cardiac toxicity are unclear. In the present study, our results demonstrated that DOX treatment significantly reduced LVEDV, SV, and EF, but increased serum BNP levels, HW/BW, liver WW/DW, and lung WW/DW compared with those in control group, whereas treatment with both ALA and DOX effectively prevented DOX-induced changes. In addition, histopathological examination demonstrated that rats in DOX group developed severe myocardial necrosis, which was significantly improved by ALA pretreatment. Thus, these findings suggested that ALA can attenuate DOX-induced cardiotoxicity in rats.

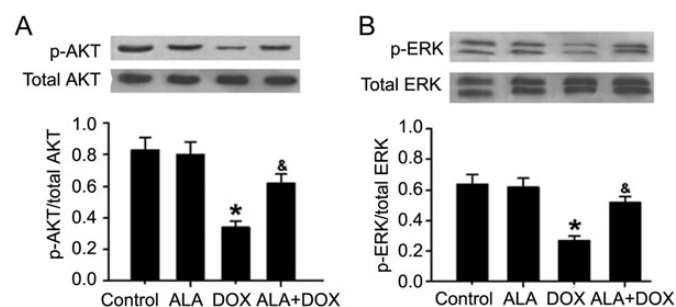


Figure 6 Effects of ALA on phosphorylation of AKT and ERK induced by DOX in cardiac tissues. The levels of p-AKT (A) and p-ERK (B) were examined by western blot analysis in cardiac tissues. Values are presented as the ratio of p-specific kinases to total kinases. Data were represented as the mean \pm SD ($n = 10$). * $P < 0.05$ vs. control group. Control, control group; ALA, α -linolenic acid group; DOX, doxorubicin group; ALA + DOX group, α -linolenic acid plus doxorubicin group. [&] $P < 0.05$ vs. DOX group.

Cytosolic enzymes such as CK-MB and LDH function as sensitive indices to evaluate the severity of myocardial injury. Increased activities of these enzymes in serum are indicative of cellular damage, loss of functional integrity, and/or permeability of cell membrane [27]. As a component of the troponin complex of muscle cells, cTnI has been shown to be a highly sensitive and specific marker of myocardial cell injury. A previous study has revealed that it is feasible to use serum cTnI levels for monitoring the extent of DOX-induced cardiac damage assessed by both non-invasive echocardiographic detection and histological changes in rats [28]. The present study demonstrated that the activities of CK-MB and LDH and serum cTnI levels are significantly increased in DOX group compared with control group. In contrast, ALA pretreatment significantly inhibited DOX-induced increases in these parameters, thereby further supporting that ALA has cardioprotective properties.

Oxidative stress represents one of the major cellular responses to toxic insults. Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipid. Since the major components of biological membranes are lipids, their peroxidation can result in cell damage and death [29]. MDA is an end-product of lipid breakdown caused by oxidative stress, and its content in tissues is considered to be a good indicator of radical-induced lipid peroxidation [30,31]. Our results exhibited a significant increase of cardiac MDA content in DOX-treated rats, suggesting the primary role of oxidative stress in DOX cardiotoxicity. These data are in agreement with earlier studies [32]. However, pretreatment with ALA significantly inhibited the elevated level of MDA compared with DOX-treated rats, indicating anti-oxidative activity of ALA in DOX-induced cardiotoxicity.

The quinone moiety of DOX undergoes a one-electron reduction that is catalyzed by NAD(P)H reductase to produce

a semiquinone free-radical intermediate, which regenerates the parent quinone by reacting with oxygen to form superoxide anions and hydrogen peroxide [33]. The ROS leads to depletion of the endogenous antioxidant system and oxidative injury. SOD, GSH-Px, and CAT constitute the first line of cellular defense against ROS, because they can decompose superoxide anions and hydrogen peroxide before their interaction to form the more reactive hydroxyl radical [34]. In this study, the lower activities of SOD, GSH-Px, and CAT were found in cardiac tissues of DOX-treated rats compared with control rats, which was consistent with the previous report [32]. The decreased activities of these enzymes might be attributed to their increased utilization for scavenging ROS [35]. However, co-treatment with ALA and DOX significantly raised the activities of SOD, GSH-Px, and CAT compared with DOX group. Moreover, pretreatment of ALA further promoted DOX-induced elevation of *SOD* gene transcription. Thus, enhancement of endogenous antioxidant enzyme activities may be one of the important mechanisms for protective effects of ALA against DOX cardiotoxicity.

Nrf2 has been described as a transcription factor that functions as a major regulator of the adaptive response to exogenous and endogenous oxidative stresses. The binding of Keap1 to Nrf2 leads to the sequestration of Nrf2 in the cytoplasm and the enhancement of Nrf2 degradation by proteasomes. It has already been reported that disruption of Keap1 repression is sufficient for activation of Nrf2 [36]. Han *et al.* [23] found that naringenin-7-*O*-glucoside isolated from *Dracocephalum rupestre* Hance can prevent cardiomyocytes from DOX-caused toxicity by Nrf2-dependent induction of SOD and CAT. In another study, Keap1/Nrf2 signaling pathway has been shown to play a critical role in suppression of oxidative cardiac fibroblast injury [37]. In the present study, we found that ALA pretreatment further promotes DOX-induced decrease in the cytosolic Keap1 abundance and increases the nuclear Nrf2 level. Thus, we presumed that activation of Keap1/Nrf2 signaling is likely to be, at least partly, responsible for the protective effects of ALA against DOX-induced cardiotoxicity. However, a further detailed study is needed to confirm this mechanism.

Apoptosis is a complicated process of deliberate suicide by a cell in a multi-cellular organism, which is involved in an orchestrated series of biochemical events leading to a characteristic cell morphology and death. Our results revealed that DOX could induce cardiomyocyte apoptosis as evaluated by TUNEL staining analysis, suggesting that apoptosis is one of the major contributors of DOX-imposed cardiac toxicity. It has already been reported that blocking the apoptosis process can prevent the loss of contractile cells and minimize myocardial injury after DOX injection [38,39]. In the present study, we found that ALA significantly attenuates DOX-induced cardiomyocyte apoptosis, implying that inhibition of apoptosis contributes to the mechanism

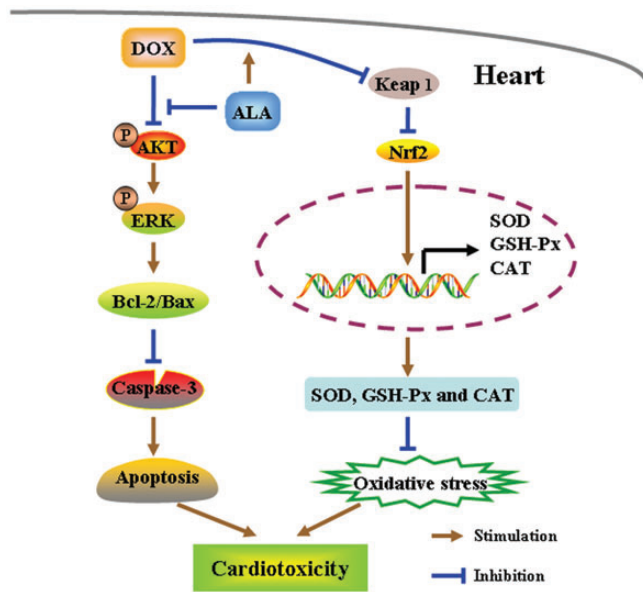


Figure 7 Schematic representation of the mechanisms responsible for the protective effects of ALA against DOX-induced cardiotoxicity. ALA pretreatment blocks DOX-mediated down-regulation of Bcl-2 expression and up-regulation of Bax expression via AKT/ERK pathway, which in turn prevents activation of caspase-3 and cardiomyocyte apoptosis. On the other hand, ALA administration further promotes DOX-exerted nuclear translocation of Nrf2 by inhibiting Keap1. This leads to increased synthesis and secretion of SOD, GSH-Px, and CAT, thereby counteracting oxidative stress. Together, anti-oxidative stress and anti-apoptotic properties of ALA contribute to its protection against cardiotoxicity induced by DOX. P, phosphorylation.

by which ALA restrains the extent of DOX-induced toxicity to myocardium.

A number of studies have demonstrated that if Bax homodimers predominate, cell death will occur, but when Bcl-2 and Bax heterodimerization prevails, cells can survive [40]. Therefore, the ratio of Bcl-2 to Bax may be a key regulator of apoptosis. It has been reported that the activation of AKT/ERK pathway leads to the increased ratio of Bcl-2 to Bax and thereby inhibits apoptosis [41]. In addition, the low ratio of Bcl-2 to Bax can activate caspase-3, which causes cleavage of cytoskeletal and nuclear proteins, and nucleosomal fragmentation [42]. In this study, we demonstrated that DOX up-regulated Bax expression, induced caspase-3 activation, and down-regulated the expression of Bcl-2, p-AKT, and p-ERK, which were blocked by ALA pretreatment. Collectively, results from these series of experiments suggested that ALA prevents DOX-induced cardiomyocyte apoptosis, to a large extent, through regulation of Bcl-2, Bax, and caspase-3 via activating AKT/ERK pathway.

In summary, our results suggested that ALA exerts significant protective effects against DOX-induced cardiotoxicity in rats and the underlying mechanisms may be associated with the enhancement of antioxidant defense system through activating Keap1/Nrf2 pathway and anti-

apoptosis through regulating Bax, Bcl-2, and caspase-3 via AKT/ERK pathway (Fig. 7). Hence, this polyunsaturated fatty acid may be a promising cardioprotective agent against DOX cardiomyopathy in clinical practice.

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References

- 1 Ferreira AL, Matsubara LS and Matsubara BB. Anthracycline-induced cardiotoxicity. *Cardiovasc Hematol Agents Med Chem* 2008, 6: 278–281.
- 2 Volkova M and Russell R, III. Anthracycline cardiotoxicity: prevalence, pathogenesis and treatment. *Curr Cardiol Rev* 2011, 7: 214–220.
- 3 Octavia Y, Tocchetti CG, Gabrielson KL, Janssens S, Crijns HJ and Moens AL. Doxorubicin-induced cardiomyopathy: from molecular mechanisms to therapeutic strategies. *J Mol Cell Cardiol* 2012, 52: 1213–1225.
- 4 Lai HC, Yeh YC, Wang LC, Ting CT, Lee WL, Lee HW and Wang KY, *et al.* Propofol ameliorates doxorubicin-induced oxidative stress and cellular apoptosis in rat cardiomyocytes. *Toxicol Appl Pharmacol* 2011, 257: 437–448.
- 5 Rashikh A, Ahmad SJ, Pillai KK, Kohli K and Najmi AK. Aliskiren attenuates myocardial apoptosis and oxidative stress in chronic murine model of cardiomyopathy. *Biomed Pharmacother* 2012, 66: 138–143.
- 6 Bordoni A, Biagi P and Hrelia S. The impairment of essential fatty acid metabolism as a key factor in doxorubicin-induced damage in cultured rat cardiomyocytes. *Biochim Biophys Acta* 1999, 1440: 100–106.
- 7 Hrelia S, Bordoni A and Biagi PL. Role of gamma-linolenic acid in counteracting doxorubicin-induced damage in cultured rat cardiomyocytes. *Prostaglandins Leukot Essent Fatty Acids* 2001, 64: 139–145.
- 8 Hrelia S, Bordoni A, Angeloni C, Leoncini E, Toschi TG, Lercker G and Biagi PL. Green tea extracts can counteract the modification of fatty acid composition induced by doxorubicin in cultured cardiomyocytes. *Prostaglandins Leukot Essent Fatty Acids* 2002, 66: 519–524.
- 9 Huan ML, Zhou SY, Teng ZH, Zhang BL, Liu XY, Wang JP and Mei QB. Conjugation with alpha-linolenic acid improves cancer cell uptake and cytotoxicity of doxorubicin. *Bioorg Med Chem Lett* 2009, 19: 2579–2584.
- 10 Huan M, Cui H, Teng Z, Zhang B, Wang J, Liu X and Xia H, *et al.* *In vivo* anti-tumor activity of a new doxorubicin conjugate via alpha-linolenic acid. *Biosci Biotechnol Biochem* 2012, 76: 1577–1579.
- 11 Pan A, Chen M, Chowdhury R, Wu JH, Sun Q, Campos H and Mozaffarian D, *et al.* alpha-Linolenic acid and risk of cardiovascular disease: a systematic review and meta-analysis. *Am J Clin Nutr* 2012, 96: 1262–1273.
- 12 Kukoba TV, Shysh AM, Moibenko OO, Kotsiuruba AV and Kharchenko OV. The effects of alpha-linolenic acid on the functioning of the isolated heart during acute myocardial ischemia/reperfusion. *Fiziol Zh* 2006, 52: 12–20.
- 13 Xie N, Zhang W, Li J, Liang H, Zhou H, Duan W and Xu X, *et al.* alpha-Linolenic acid intake attenuates myocardial ischemia/reperfusion injury through anti-inflammatory and anti-oxidative stress effects in diabetic but not normal rats. *Arch Med Res* 2011, 42: 171–181.
- 14 Oh SK, Yun KH, Yoo NJ, Kim NH, Kim MS, Park BR and Jeong JW. Cardioprotective effects of alpha-lipoic acid on myocardial reperfusion injury: suppression of reactive oxygen species generation and activation of mitogen-activated protein kinase. *Korean Circ J* 2009, 39: 359–366.

- 15 Zhang ZC, Li SJ, Yang YZ, Chen RZ, Ge JB and Chen HZ. Effect of astragaloside on cardiomyocyte apoptosis in murine coxsackievirus B3 myocarditis. *J Asian Nat Prod Res* 2007, 9: 145–151.
- 16 Yu XH, Jiang HL, Chen WJ, Yin K, Zhao GJ, Mo ZC and Ouyang XP, *et al.* Interleukin-18 and interleukin-12 together downregulate ATP-binding cassette transporter A1 expression through the interleukin-18R/nuclear factor-kappaB signaling pathway in THP-1 macrophage-derived foam cells. *Circ J* 2012, 76: 1780–1791.
- 17 Fujita H, Fujishima H, Morii T, Sakamoto T, Komatsu K, Hosoba M and Narita T, *et al.* Modulation of renal superoxide dismutase by telmisartan therapy in C57BL/6-Ins2(Akita) diabetic mice. *Hypertens Res* 2012, 35: 213–220.
- 18 Fontes JP, Goncalves M and Ribeiro VG. Serum markers for ischemic myocardial damage. *Rev Port Cardiol* 1999, 18: 1129–1136.
- 19 Berbecki J, Mitosek-Szewczyk K, Kurzepa J, Nastaj M, Lobejko K and Stelmasiak Z. Processes of free radical lipid peroxidation with a particular regard to the role of paraoxonase-1 in the pathogenesis of multiple sclerosis. *Wiad Lek* 2011, 64: 31–36.
- 20 Zhang L, Ma J and Liu H. Protective effect of ischemic postconditioning against ischemia reperfusion-induced myocardium oxidative injury in IR rats. *Molecules* 2012, 17: 3805–3817.
- 21 Yousef MI, Saad AA and El-Shennawy LK. Protective effect of grape seed proanthocyanidin extract against oxidative stress induced by cisplatin in rats. *Food Chem Toxicol* 2009, 47: 1176–1183.
- 22 Kaspar JW, Niture SK and Jaiswal AK. Nrf2:INrf2 (Keap1) signaling in oxidative stress. *Free Radic Biol Med* 2009, 47: 1304–1309.
- 23 Han X, Pan J, Ren D, Cheng Y, Fan P and Lou H. Naringenin-7-O-glucoside protects against doxorubicin-induced toxicity in H9c2 cardiomyocytes by induction of endogenous antioxidant enzymes. *Food Chem Toxicol* 2008, 46: 3140–3146.
- 24 Sahin K, Orhan C, Akdemir F, Tuzcu M, Iben C and Sahin N. Resveratrol protects quail hepatocytes against heat stress: modulation of the Nrf2 transcription factor and heat shock proteins. *J Anim Physiol Anim Nutr (Berl)* 2012, 96: 66–74.
- 25 Breckenridge DG and Xue D. Regulation of mitochondrial membrane permeabilization by BCL-2 family proteins and caspases. *Curr Opin Cell Biol* 2004, 16: 647–652.
- 26 Singla DK, Ahmed A, Singla R and Yan B. Embryonic stem cells improve cardiac function in doxorubicin-induced cardiomyopathy mediated through multiple mechanisms. *Cell Transplant* 2012, 21: 1919–1930.
- 27 Rohilla A, Khan MU and Khanam R. Cardioprotective potential of simvastatin in the hyperhomocysteinemic rat heart. *J Adv Pharm Technol Res* 2012, 3: 193–198.
- 28 Bertinchant JP, Polge A, Juan JM, Oliva-Lauraire MC, Giuliani I, Marty-Double C and Burdy JY, *et al.* Evaluation of cardiac troponin I and T levels as markers of myocardial damage in doxorubicin-induced cardiomyopathy rats, and their relationship with echocardiographic and histological findings. *Clin Chim Acta* 2003, 329: 39–51.
- 29 Winczura A, Zdzalik D and Tudek B. Damage of DNA and proteins by major lipid peroxidation products in genome stability. *Free Radic Res* 2012, 46: 442–459.
- 30 Gul M, Ayan M, Seydanoglu A, Cander B, Girisgin S, Erayman I and Erdem S. The effect of N-acetyl cysteine on serum glutathione, TNF-alpha and tissue malondialdehyde levels in the treatment of sepsis. *Ulus Travma Acil Cerrahi Derg* 2011, 17: 293–297.
- 31 Giera M, Lingeman H and Niessen WM. Recent advancements in the LC- and GC-based analysis of malondialdehyde (MDA): a brief overview. *Chromatographia* 2012, 75: 433–440.
- 32 Elberry AA, Abdel-Naim AB, Abdel-Sattar EA, Nagy AA, Mosli HA, Mohamadin AM and Ashour OM. Cranberry (*Vaccinium macrocarpon*) protects against doxorubicin-induced cardiotoxicity in rats. *Food Chem Toxicol* 2010, 48: 1178–1184.
- 33 Minotti G, Menna P, Salvatorelli E, Cairo G and Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. *Pharmacol Rev* 2004, 56: 185–229.
- 34 Wattanapitayakul SK and Bauer JA. Oxidative pathways in cardiovascular disease: roles, mechanisms, and therapeutic implications. *Pharmacol Ther* 2001, 89: 187–206.
- 35 Xu J and Li Y. Effects of salidroside on exhaustive exercise induced oxidative stress in rats. *Mol Med Rep* 2012, 6: 1195–1198.
- 36 Wakabayashi N, Itoh K, Wakabayashi J, Motohashi H, Noda S, Takahashi S and Imakado S, *et al.* Keap1-null mutation leads to postnatal lethality due to constitutive Nrf2 activation. *Nat Genet* 2003, 35: 238–245.
- 37 Zhu H, Itoh K, Yamamoto M, Zweier JL and Li Y. Role of Nrf2 signaling in regulation of antioxidants and phase 2 enzymes in cardiac fibroblasts: protection against reactive oxygen and nitrogen species-induced cell injury. *FEBS Lett* 2005, 579: 3029–3036.
- 38 Lv X, Yu X, Wang Y, Wang F, Li H, Lu D and Qi R, *et al.* Berberine inhibits doxorubicin-triggered cardiomyocyte apoptosis via attenuating mitochondrial dysfunction and increasing Bcl-2 expression. *PLoS One* 2012, 7: e47351.
- 39 Chen YL, Loh SH, Chen JJ and Tsai CS. Urotensin II prevents cardiomyocyte apoptosis induced by doxorubicin via Akt and ERK. *Eur J Pharmacol* 2012, 680: 88–94.
- 40 Tang XQ, Ren YK, Zhou CF, Yang CT, Gu HF, He JQ and Chen RQ, *et al.* Hydrogen sulfide prevents formaldehyde-induced neurotoxicity to PC12 cells by attenuation of mitochondrial dysfunction and pro-apoptotic potential. *Neurochem Int* 2012, 61: 16–24.
- 41 Yin Y, Guan Y, Duan J, Wei G, Zhu Y, Quan W and Guo C, *et al.* Cardioprotective effect of danshensu against myocardial ischemia/reperfusion injury and inhibits apoptosis of H9c2 cardiomyocytes via Akt and ERK1/2 phosphorylation. *Eur J Pharmacol* 2013, 699: 219–226.
- 42 Shi K, Wang D, Cao X and Ge Y. Endoplasmic reticulum stress signaling is involved in mitomycin C (MMC)-induced apoptosis in human fibroblasts via PERK pathway. *PLoS One* 2013, 8: e59330.