Original Article
Astragalus polysaccharide improves cardiac function in doxorubicin-induced cardiomyopathy through ROS-p38 signaling

Liangliang Zhou[1], Lanping Chen[1], Jing Wang[2], Yijun Deng[1]

[1]Department of Critical Care Medicine, Yancheng City, No. 1 People's Hospital, Yancheng, Jiangsu, China; [2]Department of Cardiology, Jinling Hospital, Nanjing, 210002, China

Abstract: Doxorubicin (DOX) is widely used as an antitumor agent, but it is significantly challenged by clinical workers due to the severe and acute cardiotoxicity. Astragalus polysaccharide (APS) is characterized by an anti-inflammation and anti-oxidant features. In the current study, we explored the effects and specific mechanisms of APS on DOX-induced-cardiomyopathy in mouse primary myocardial cells. To explore the effect of DOX on ROS production, DHE staining and flow cytometry analysis were used in primary cardiomyocytes treated with 1 μM DOX for 24 h. MTT assay was applied to determine the effect of DOX on cell viability. The effects of DOX on rat cardiomyocytes apoptosis by Hoechst staining and annexin V-PI staining, while caspase3 activity was determined using an assay kit. Two-dimensional echocardiography of rats was performed to determine left ventricular fraction and relative wall thickness. Activation of p38 and Akt was analyzed using western blot. ROS production was significantly enhanced by DOX stimulation in primary cardiomyocytes. DOX reduced rat cardiomyocytes viability in a time- and dose-dependent manner. DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3. Cardiac function was significantly impaired by enhanced p38 activation. APS treatment reduced DOX-induced rat cardiomyocytes apoptosis by decreasing ROS production. To conclude, APS reduced DOX-induced cell apoptosis and ROS production by reduced activation of p38 signaling pathway.

Keywords: APS, DOX, cardiac cell apoptosis, ROS, p38 signaling

Introduction

Doxorubicin (DOX) is widely used as an antitumor agent in a variety of cancers due to the antineoplastic and antibiotic characteristics [1, 2]. However, DOX is significantly challenged by clinical workers due to the severe and acute cardiotoxitity that finally leads to the irreversible chronic cardiomyopathy and heart failure. In tons of animal studies, DOX induced cardiomyopathy (DIC) has been widely reported [3]. But the specific mechanism is still not clearly DIC elucidated. Studies indicate that mulifactors and mechanisms are involved in the pathology [3]. Among these factors, oxidative stress may play a key role in DIC. Contractile dysfunction and rhythm disturbances of DIC result in the congestive heart failure in a dose dependent manner [1]. And progression of heart failure mainly involves the death of cardiac myocytes and non-myocyte myocardial cells as well as myofibril loss and fibrosis [4, 5]. Moreover, activation of matrix metalloproteinases (MMPs) induces extracellular matrix (ECM) degradation thereby increasing the collagen synthesis [5]. In previous studies, erythropoietin and antioxidants treatment demonstrates strong inhibiting capabilities of DIC-related apoptosis [6]. Since DIC is a major public health problem, necessary treatment methods should be explored.

In clinical practices, many approaches have been applied to reduce the severe side effects. However, there are still many problems [7]. Through DNA intercalation and Topoisomerase II inhibition, DOX can significantly kill cancer cells. However, in the heart failure, DOX has been showed to enhance oxidative stress by increased reactive oxygen species (ROS), such as O_2^-, OH^- and H_2O_2 [8]. From this perspective, cardiac myocytes death can be avoided by blocking the cardiomyocyte death pathways,

which may improve DOX-induced side effects. And tons of studies find that supplementation of antioxidants in animals significantly improves DOX-induced heart failure [3]. However, clinical trials with vitamin E, an antioxidant, did not demonstrate obvious improvement [9]. Therefore, further study is needed to explore DOX induced cardiomyocyte death from the perspective of antioxidant-based therapy.

Astragalus polysaccharide (APS) is the major monomer that is extracted from Huang qi (Radix Astragali seu Hedysari). APS has long been used as an anti-inflammation and anti-oxidant herbal prescription in traditional Chinese medicine [10, 11]. In previous studies, APS has been widely reported to possess potent immunomodulatory activity [12]. One of the mechanisms of APS-related immunomodulatory activity is due to its anti-oxidative and anti-inflammatory capabilities. In the current study, we explored the effects and specific mechanisms of APS on DIC in mouse primary myocardial cells.

Materials and methods

Primary cardiomyocyte culture

Primary cardiomyocytes from rat neonatal hearts were isolated as described [13]. Animal protocol was approved by the Jinling Hospital. Briefly, hearts were isolated and digested with collagenase type II (Worthington) solution. After digestion, the cells were preplated for 2 hr to collect cardiomyocytes. Then, the attached cells were considered as non-myocytes and discarded. And the unattached cells were primarily cardiomyocytes.

Study protocols -in vivo

Eight-week-old male Sprague-Dawley rats were obtained from the fourth affiliated hospital of Harbin medical University. Then, the SD rats were injected with 3 mg/kg DOX (n = 4) or saline (n = 4) through the tail vein weekly for 5 weeks [14]. After injection with DOX or saline, the rats were explored respectively at week 0, 2 weeks and 4 weeks. The cardiac function was evaluated with echocardiography. After that, the hearts were excised and the proteins were extracted.

Two-dimensional echocardiography

Two-dimensional echocardiography was performed with certain modifications according to the previously reported methods [15]. On the day of evaluation, sodium pentobarbital (50 mg/kg) was used to anesthesize the rats. After shaving the chest, two-dimensional echocardiography was conducted using the echocardiographic systems (model SSD-900; Aloka, Tokyo) and 7.5 MHz probe (UST-987-7.5, Aloka). The M-mode echocardiograms of the left ventricle (LV) were determined at the papillary muscle level. All the index, including end-diastolic posterior wall thickness, end-diastolic and end-systolic internal diameters of the LV, were explored by a single observer. The follow formular was applied to determine the relative wall thickness (RWT):

RWT = 2 × LVPWTd/LVDd, LVPWTd refers to an end-diastolic posterior wall thickness of LV, and LVDd means an end-diastolic internal diameter of LV.

The formular was applied to determin fractional shortening (FS):

FS = 100 × (LVDd - LVDs)/LVDd; LVDs equals to an end-systolic internal diameter of the LV.

Dimethyl thiazolyl diphenyl tetrazolium (MTT) assay

Cell viability was determined by a colormetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, sigma). To determine the impacts of DOX on cell viability, primary cardiomyocytes were cultured at approximately 70% confluency and starved in serum-free DMEM (SF-DMEM) (Life Technologies, Inc.) overnight. Then, 1 nM, 10 nM, 100 nM, 1 µM, and 10 µM DOX was preincubated with primary cardiomyocytes for 24 h. After drug treatment, the cells were cultured in fresh medium including 0.5 mg/mL MTT for 4 h. Then, DMSO was added into the wells to dissolve the blue formazan products and the density was determined spectrophotometrically at a wavelength of 550 nm. Besides, the cells were preincubated with 1 µM DOX for 8, 16, 24, 48 h and cell viability was determined in the same method as described. Each experiment was independently performed at least 3 times.

Hoechst 33258 staining

Primary cardiomyocytes (1×10^5 cells per well) were cultured in six-well tissue culture plates. The cells were incubated in serum-free DMEM medium for 16 h at 70-80% confluence. Then, 1 µM DOX was added to the fresh medium and preincubated with the cells for 48 h. After DOX treatment, the medium was removed, and the cells were washed three times with cold PBS and then fixed with 4% formaldehyde (Zhongshan Technology) in PBS for 20 min at room temperature. Then, the cells were washed three times with cold PBS and stained with Hoechst 33258 (10 µg/ml) (50 µL/slides) (Sigma) for 5 min. After staining, the cells were further rinsed with cold PBS and examined under fluorescence microscope.

Apoptosis assay

To detect the effects of DOX on primary cardiomyocytes apoptosis, the cells were treated with 1 µM DOX for 24 h with or without preincubation of 1 µM APS. After DOX treatment, the cells were washed with cold PBS for three times. Then, flow cytometry was used to dermine cell apoptosis with an Annexin-V FITC-PI Apoptosis Kit (Invitrogen, Carlsbad, CA). In summary, cells were washed with $1 \times$ PBS for three times and suspended at 2-3 $\times$ 10^6 cells/mL in 1 $\times$ Annexin-V Binding Buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Annexin-V FITC and Propidium Iodide Buffer were added to the cells, which were then incubated at room temperature for 15 minutes in the dark. The cells without any treatment were used as internal control. After incubation, the cells were filtered by a filter screen and the cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ) within 1 h of staining.

Determination and quantification of ROS

Cells were cultured on slides in six-well chamber at 60% confluence. Two days later, the slides were washed with cold PBS for three times. And then the slides were treated with 5 µM DHE (Vigorous Biotechnology Beijing Co., Ltd) in serum-free DMEM F-12 medium for 30 min at 37°C in darkness. The cells were fixed in 4% paraformaldehyde for 30 min at RT. The slides were washed with cold PBS for three times and mounted. Immunofluorescence images were captured by fluorescence microscopy. To quantify the intracellular ROS, relative fluorescence intensities were analyzed with flow cytometry (Becton-Dickinson) in the primary cardiomyocytes.

Measurement of caspase-3 activities

To measure the changes of caspase-3 activity, an assay kit (Medical & Biological Laboratory, Nagoya) was applied (22) through exploring the cleavage of the substrate 7-amino-4-trifluoromethyl coumarin conjugated to Asp-Glu-Val-Asp (DEVD-AFC). Firstly, 5 $\times$ 10^5 cardiomyocyte cells were harvested and treated with the substrate for 1 h at 37°C. Then, the intensity of fluorescence was determined using a spectrophotometer (Bio Lumin 960; Molecular Dynamics, Sunnyvale, CA, USA). The activity was determined in comparison with the control.

Protein extraction, western blotting and antibodies

Cellular proteins were extracted using RIPA buffer (SolarBio, 50 mM Tris/HCl, pH 7.4, 150 mM NaCl 1% (v/v) NP-40, 0.1%(w/v) SDS) with 1% (v/v) PMSF (SolarBio), 0.3% (v/v) protease inhibitor (Sigma) and 0.1% (v/v) phosphorylated proteinase inhibitor (Sigma). Lysates were collected for total protein after centrifugation. BCA protein assay kit (Pierce) was used to determine the protein concentration. 15 µg of protein was separated on an SDS-PAGE gel (10% (v/v) polyacrylamide) and transferred onto a PVDF membrane. After blocking with 8% (w/v) milk in PBST for 2 hr at room temperature, the membranes were then incubated with primary antibodies against GAPDH, cleaved-caspase3, p-Akt, p-p38, Akt and p38 (Cell Signaling) overnight at 4°C. Then, the membranes were washed with TBST for three times. And the membranes were incubated in HRP-conjugated goat anti-rabbit or HRP-conjugated mouse anti-goat IgG (Abmart, all at a 1:5000 dilution) for 2 hr at room temperature and then washed. Enhanced chemiluminescence (Millipore) was used to determine the protein concentrations according to the manufacturer's recommendations. The relative contents of protein was normalized against GAPDH.

Statistical analysis

Data were presented as mean ± SE from 3 independent experiments. Statistical analysis
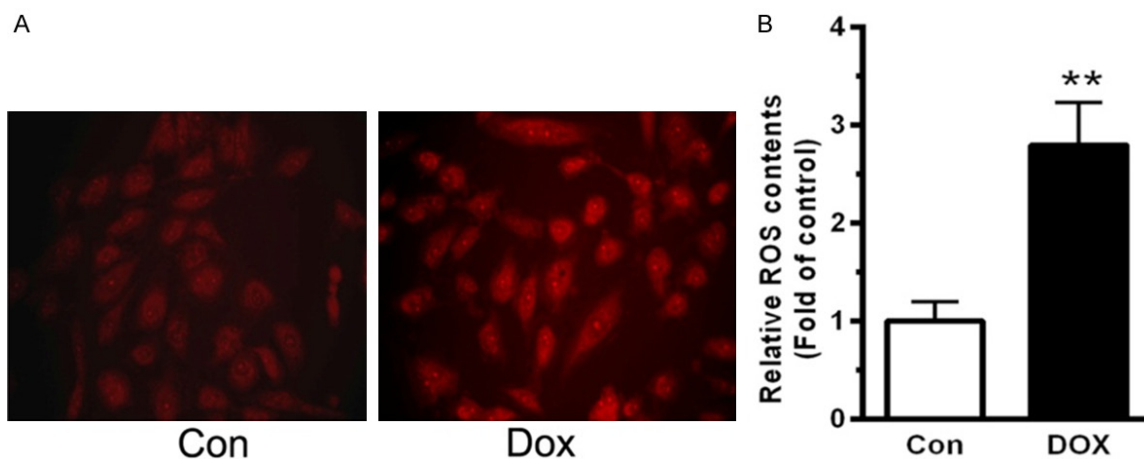
Figure 1. DOX significantly enhanced ROS production in primary rat cardiomyocytes. Primary rat cardiomyocytes. were exposed to 1 µM DOX for 10 h. ROS production was determined using DHE staining (A) and flow cytometry (B). Data represent the means ± SEM, n = 3 independent experiments. **$P<0.01$, versus control.
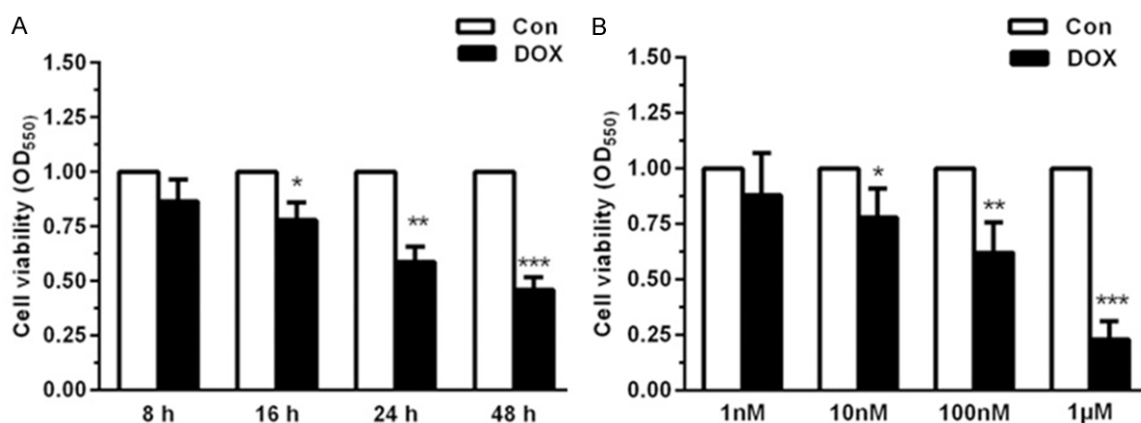

Figure 2. DOX inhibited primary rat cardiomyocytes viability in a time- and dose-dependent manner. Rat cardiomyocytes were preincubated with 1 µM DOX for 8, 16, 24, 48 hrs. A. Meanwhile, when the cells were preincubated with 1 nM, 10 nM, 100 nM, and 1 µM DOX for 24 hrs, B. Cell viability was analyzed by MTT assay, respectively. Data represent the means ± SEM, n = 6 independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control.

was carried out with Student's t test. $P < 0.05$ was considered as statistically significant difference.

Results

*DOX significantly enhanced ROS production in primary rat cardiomyocytes*

To explore the effect of DOX on ROS production, primary cardiomyocytes from rat neonatal hearts were treated with 1 µM DOX for 24 h. DHE staining showed that DOX significantly enhanced ROS production (**Figure 1A**). Furthermore, flow cytometry analysis revealed that DOX increased more than 2 fold of ROS production compared with control (**Figure 1B**). These data indicated that ROS production was significantly

enhanced by DOX stimulation in primary cardiomyocytes.

DOX inhibited primary rat cardiomyocytes viability in a time- and dose-dependent manner

To determine the effect of DOX on cell viability, rat cardiomyocytes were preincubated with 1 µM DOX for 8, 16, 24, 48 hrs. As shown in **Figure 2A**, preincubation with DOX decreased cell viability by 32% and 45% at 24 h and 48 h, respectively. Meanwhile, when the cells were preincubated with 1 nM, 10 nM, 100 nM, and 1 µM DOX for 24 hrs, cell viability was reduced by 30% and 74% at 100 nM, and 1 µM, respectively (**Figure 3B**). These results demonstrated that DOX reduced rat cardiomyocytes viability in a time- and dose-dependent manner.
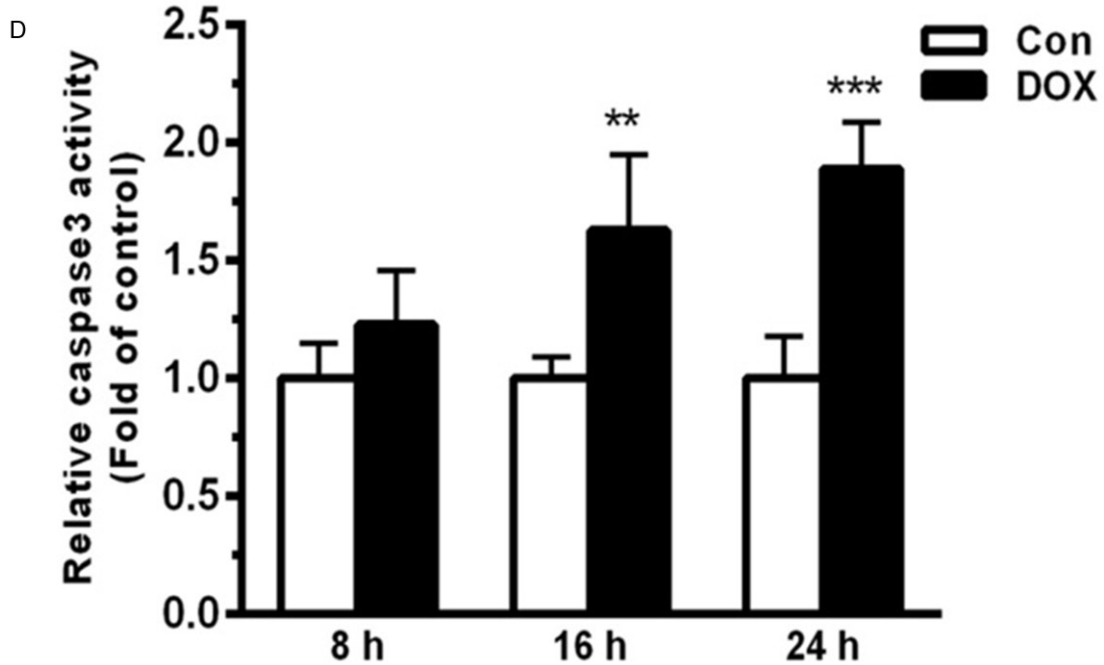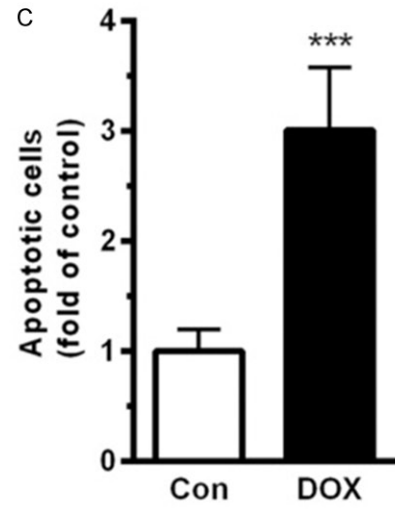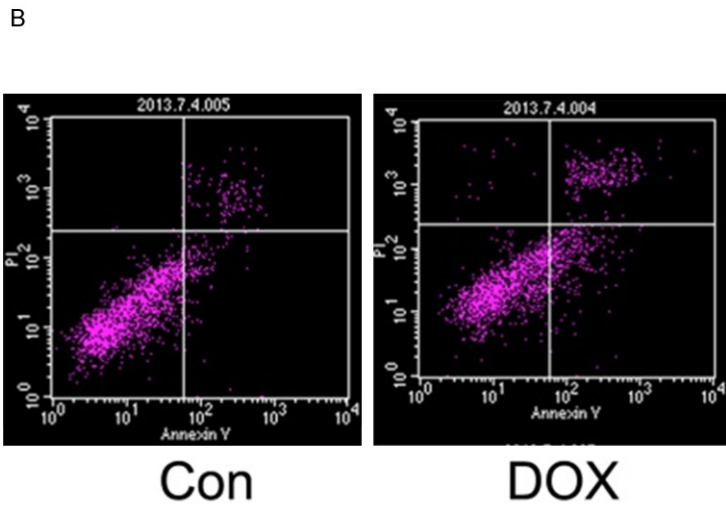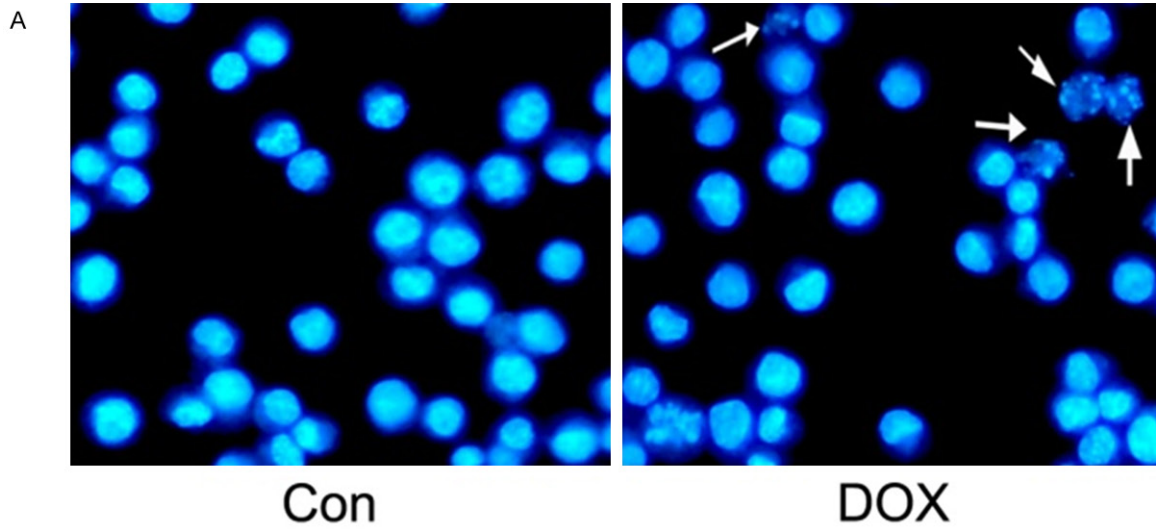
Figure 3. DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3. A. Rat cardiomyocytes were exposed to 1 μM DOX for 24 hrs and cell apoptosis was evaluated by Hoechst staining. B. Cell apoptosis was identified using flow cytometry. C. Statistical analysis of apoptotic cells that were treated with 1 μM DOX for 24 hrs. D. The activity of caspase-3 was determined relative to the level of the control. Data represent the means ± SEM, n = 3 independent experiments. **$P<0.01$, ***$P<0.001$ versus control.
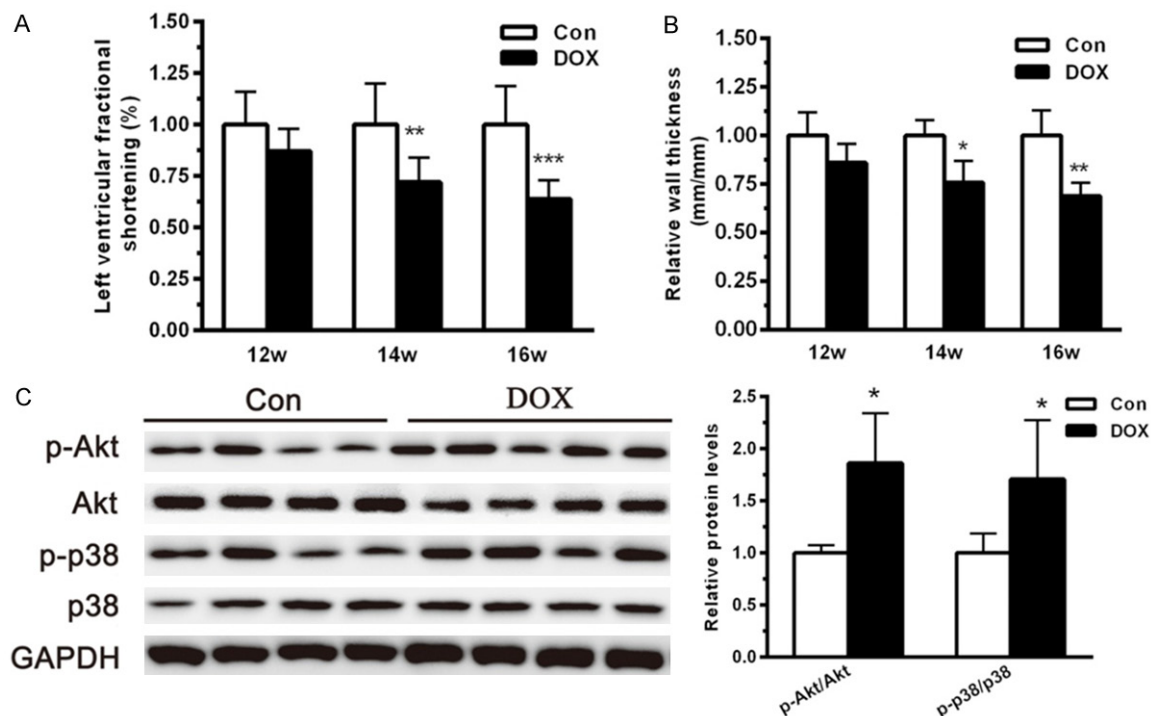

Figure 4. Rats cardiac function was significantly impaired by reduced energy metabolism. The left ventricular fractional shortening (A) and the relative wall thickness (B) of rats with or without doxorubicin (DOX). (C) Activation of p38 and Akt was analyzed in rats injected with 3 mg/kg DOX (n = 4) or saline (n = 4) through the tail vein for 5 weeks. Data represent the means ± SEM, n = 3 independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control.

DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3

We next observed the effects of DOX on rat cardiomyocytes apoptosis by Hoechst staining. As shown in **Figure 3A**, cell apoptosis was obvious in rat cardiomyocytes that were treated with 1 μM DOX for 24 hrs when compared with the control (**Figure 3A**). Furthermore, rat cardiomyocytes apoptosis was also analyzed using Annexin V-PI staining. As analyzed by flowcytometry, rat cardiomyocytes apoptosis was significantly enhanced by 1 μM DOX treatment, which was increased by nearly two fold (**Figure 3C**). Furthermore, the activity of caspase-3 in DOX-treated rat cardiomyocytes was significantly increased over untreated cells at 16 h and 24 h (**Figure 3D**). Together these data indicated that DOX induced apoptosis in rat cardiomyocytes via activation of caspase-3.

DOX decreased cardiac function with enhanced p38 activation

In rats with DOX, FS was significantly decreased at age 14 weeks (2 weeks after the end of DOX-administration) and was further markedly diminished at age 16 weeks (**Figure 4A**). The changes in RWT of the LV, as assessed by echocardiography, are shown in **Figure 4B**. In rats with DOX, RWT was significantly decreased at age 14 weeks and was still further reduced at age 16 weeks (**Figure 3B**). Furthermore, we also examined the energy metabolism status in rats with DOX or saline. As shown in **Figure 3C**, when rats were injected with 3 mg/kg DOX (n = 4) or saline (n = 4) through the tail vein for 5 weeks, caspase3 was significantly activated at 16 weeks (**Figure 4C**). And the activation of p38 was significantly enhanced. Interestingly, the phosphorylation of Akt was obviously enhanced
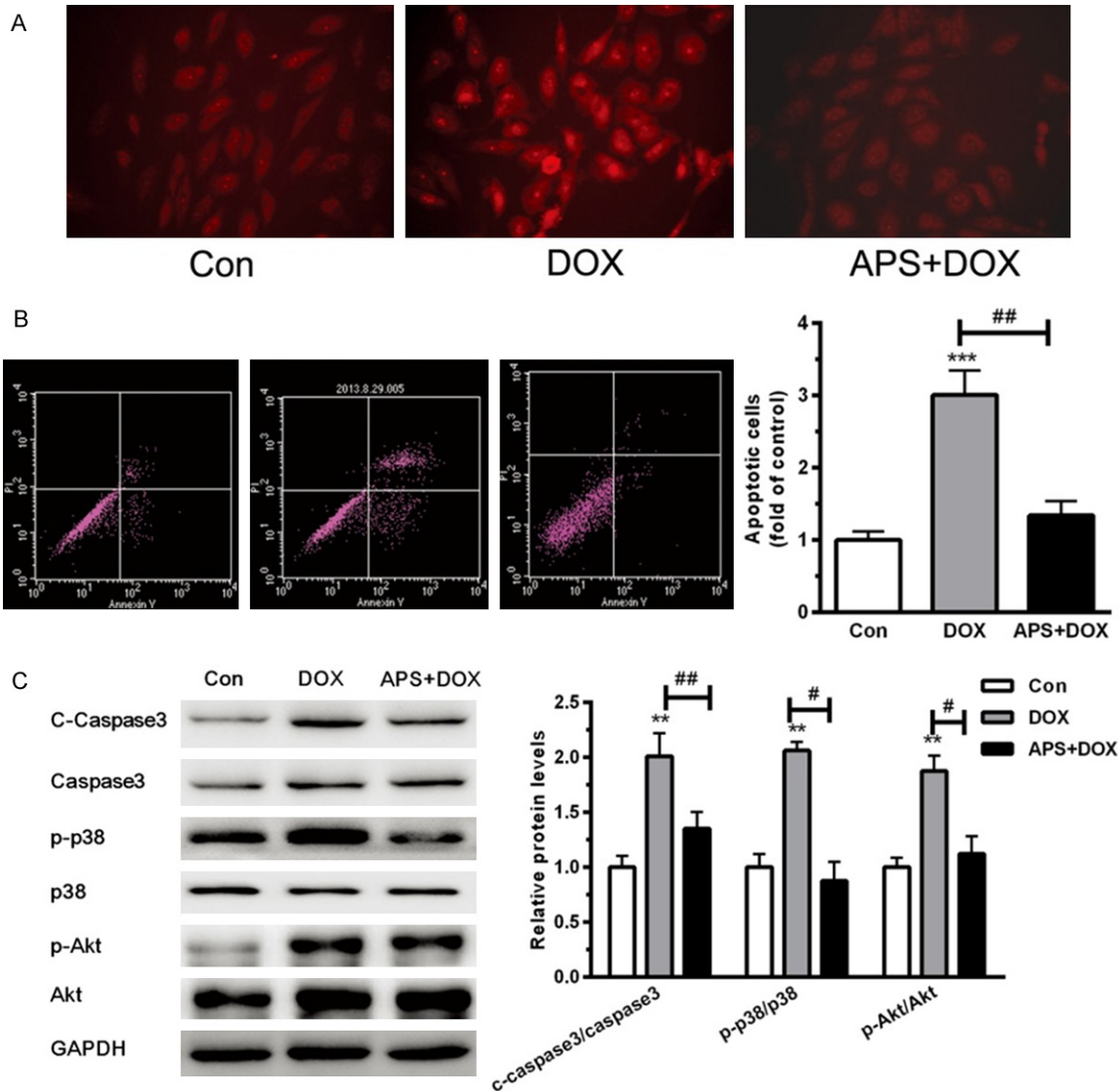
Figure 5. APS reduced DOX-induced rat cardiomyocytes apoptosis by decreasing p38 activation. A. Preincubation with 1 µM APS significantly reduced DOX-induced ROS contents. B. Cell apoptosis rate was significantly reduced when rat cardiomyocytes were pretreatment with 1 µM APS as analyzed by flow cytometry. C. Westen blot analysis was applied to explore caspase-3, p38 and Akt activation in rat cardiomyocytes treated by DOX with or without preincubation of 1 µM APS. Data represent the means ± SEM, n = 3 independent experiments. *$P<0.05$, **$P<0.01$, versus control. #$P<0.05$, ##$P<0.01$ versus APS+DOX.

which suggested a potential stress response thereby protecting the heart (**Figure 4C**). These data indicated that cardiac function was significantly impaired by enhanced p38 activation.

APS treatment reduced DOX-induced rat cardiomyocytes apoptosis by decreasing ROS production

To explore the protective role of APS on DOX-induced ROS production, rat cardiomyocytes were preincubated with or without preincubation of 1 µM APS and ROS contents were determined using DHE staining. As shown in **Figure 5A**, preincubation with 1 µM APS significantly reduced DOX-induced ROS contents. Meanwhile, flow cytometry was applied to determine apoptotic cells. As shown in **Figure 5B**, cell apoptosis rate was significantly reduced when rat cardiomyocytes were pretreatment with 1 µM APS. Furthermore, in previous report, it has been suggested that p38 MAPKs and p-Akt are involved in cell apoptosis [16]. Thus, we also tested caspase-3, p38 and Akt activation in rat cardiomyocytes treated by DOX with or without preincubation of 1 µM APS. The results found

that APS preincubation significantly reduced DOX-induced caspase3 activation. In comparison, p38 phosphorylation was significantly reduced with preincubation of 1 μM APS. These data indicated that APS reduced DOX-induced rat cardiomyocytes apoptosis by decreasing p38 activation.

Discussion

In previous studies, apoptosis was significantly induced in animal models and patients of heart failure [17, 18]. Dox was formally used as an anti-tumor agent in clinical practices. However, the cardiac toxicity significantly restricted its promotion and application. In the present study, we found that DOX significantly induced ROS production and cell apoptosis in cardiomyocytes. Meanwhile, the activity of caspase-3 was significantly increased. In the rats injected with DOX, the left ventricular function had significantly impaired and the RWT was obviously reduced. These data indicated that DOX induced cardiomyocytes apoptosis mainly by increased ROS production and activation of caspase3. And it is thus suggested that apoptosis played a key role in the progression of myocardial dysfunction induced by DOX.

Oxidative stress are proved to be widely involved in cellular apoptosis [19]. Upregulation of ROS usually leads to abnormal intracellular signaling pathways and then triggers cell apoptosis [20]. Consequently, reduced ROS production prompts cell survival in a variety of cells [21]. We showed that DOX stimulated ROS formation thereby enhancing cell apoptosis. Then, we explored several different cellular signaling pathways that are suggested to be stimulated by ROS. PI3K/Akt signaling pathway was proved to significantly enhanced cell survival. In this study, when rat cardiomyocytes were treated with DOX, Akt was obviously activated, which may indicate the stress-response effect of cardiomyocytes. This result is in consistent with other studies, which all prove the stress-response effect of cardiomyocyte after abnormal stimulation [22, 23]. MAPKs are also key regulators in cell survival, which mainly include p38, ERK, and JNK [24]. In this study, we choose p38 as a representative signaling effecter in MAPK signaling pathway. Our data found that phosphorylation of p38 was significantly enhanced, which indicated impaired cell survival. And we also tested cell viability with MTT assay,

which indicated the DOX impaired cell viability in a time- and dose- dependent manner. These data indicated that DOX reduced cardiomyocytes viability through the ROS-p38-pathway.

Astragalus polysaccharide (APS) is an active component extracted from traditional Chinese medicine, which is widely used in a variety of disease models, such as diabetes, cancer, renal failure and so on. Previous studies have demonstrated the antioxidant, antihypertensive, and immunomodulatory characteristics [25, 26]. In this study, we found that preincubation with APS significantly decreased DOX-induced ROS production. Consequently, decreased oxidative stress by lowering ROS production significantly inhibited cell apoptosis. APS was found to inhibit cell apoptosis by scavenging ROS and decreasing mitochondrial permeability transition. In line with the study, we demonstrated that DOX induced ROS production and APS treatment demonstrated obvious protective effects by reducing caspase3 activation [18]. Several studies have indicated that APS can inhibit ROS-p38 activation in advanced glycation end product-stimulated macrophages [27]. Similar to these findings, our studies found that APS reduced DOX-induced cell apoptosis by reduced activation of p38 signaling pathway.

In summary, DOX induced cardiomyocytes apoptosis mainly by increased ROS production and activation of caspase3, which then play key roles in DOX-induced cardiomyopathy. Furthermore, APS worked as an anti-oxidant and anti-inflamatory agent, which significantly decreased ROS production and prevented cardiac injury.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Yijun Deng, Department of Critical Care Medicine, Yancheng City, No. 1 People's Hospital, Yancheng, Jiangsu, China. E-mail: yijundeng2015@126.com

References

[1] Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: Molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 2004; 56: 185-229.

[2] Dursun N, Taskin E, Ozturk F. Protection against adriamycin-induced cardiomyopathy by carnosine in rats: Role of endogenous antioxidants. Biol Trace Elem Res 2011; 143: 412-424.

[3] Ivanov D, Shabalov N, Petrenko Y, Shabalova N, Treskina NA. The specific characteristics of dic syndrome vary with different clinical settings in the newborn. J Matern Fetal Neonatal Med 2014; 27: 1088-92.

[4] Kumar D, Kirshenbaum L, Li T, Danelisen I, Singal P. Apoptosis in isolated adult cardiomyocytes exposed to adriamycin. Ann N Y Acad Sci 1999; 874: 156-68.

[5] Zhao Y, McLaughlin D, Robinson E, Harvey AP, Hookham MB, Shah AM, McDermott BJ, Grieve DJ. Nox2 nadph oxidase promotes pathologic cardiac remodeling associated with doxorubicin chemotherapy. Cancer Res 2010; 70: 9287-9297.

[6] Kim KH, Oudit GY, Backx PH. Erythropoietin protects against doxorubicin-induced cardiomyopathy via a phosphatidylinositol 3-kinase-dependent pathway. J Pharmacol Exp Ther 2008; 324: 160-169.

[7] Jain A, Kishore K. Doxorubicin-induced dilated cardiomyopathy for modified radical mastectomy: A case managed under cervical epidural anaesthesia. Indian J Anaesth 2013; 57: 185-187.

[8] Mukhopadhyay P, Rajesh M, Batkai S, Patel V, Kashiwaya Y, Liaudet L, Evgenov OV, Mackie K, Hasko G, Pacher P. Cb1 cannabinoid receptors promote oxidative stress and cell death in murine models of doxorubicin-induced cardiomyopathy and in human cardiomyocytes. Cardiovasc Res 2010; 85: 773-784.

[9] Brilhante Wolle CF, de Aguiar Zollmann L, Etges A, Vitalis GS, Leite CE, Campos MM. Effects of the antioxidant agent tempol on periapical lesions in rats with doxorubicin-induced cardiomyopathy. J Endod 2012; 38: 191-195.

[10] Zhang C, Zhu C, Ling Y, Zhou X, Dong C, Luo J, Liu Y. The clinical value of huangqi injection in the treatment of leucopenia: A meta-analysis of clinical controlled trials. PLoS One 2013; 8: e83123.

[11] Feng M, Yuan W, Zhang R, Fu P, Wu T. Chinese herbal medicine huangqi type formulations for nephrotic syndrome. Cochrane Database Syst Rev 2013; 6: CD006335.

[12] Zhao LH, Ma ZX, Zhu J, Yu XH, Weng DP. Characterization of polysaccharide from astragalus radix as the macrophage stimulator. Cell Immunol 2011; 271: 329-334.

[13] Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM, Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. Dev Cell 2009; 16: 233-244.

[14] Fujita N, Hiroe M, Ohta Y, Horie T, Hosoda S. Chronic effects of metoprolol on myocardial beta-adrenergic receptors in doxorubicin-induced cardiac damage in rats. J Cardiovasc Pharmacol 1991; 17: 656-661.

[15] Cittadini A, Stromer H, Katz SE, Clark R, Moses AC, Morgan JP, Douglas PS. Differential cardiac effects of growth hormone and insulin-like growth factor-1 in the rat. A combined in vivo and in vitro evaluation. Circulation 1996; 93: 800-809.

[16] Li Z, Zhang H, Chen Y, Fan L, Fang J. Forkhead transcription factor foxo3a protein activates nuclear factor kappab through b-cell lymphoma/leukemia 10 (bcl10) protein and promotes tumor cell survival in serum deprivation. J Biol Chem 2012; 287: 17737-17745.

[17] Liu M, Wu K, Mao X, Wu Y, Ouyang J. Astragalus polysaccharide improves insulin sensitivity in kkay mice: Regulation of pkb/glut4 signaling in skeletal muscle. J Ethnopharmacol 2010; 127: 32-37.

[18] Li XT, Zhang YK, Kuang HX, Jin FX, Liu DW, Gao MB, Liu Z, Xin XJ. Mitochondrial protection and anti-aging activity of astragalus polysaccharides and their potential mechanism. Int J Mol Sci 2012; 13: 1747-1761.

[19] Ghosh R, Girigoswami K, Dipanjan G. Suppression of apoptosis leads to cisplatin resistance in v79 cells subjected to chronic oxidative stress. Indian J Biochem Biophys 2012; 49: 363-370.

[20] Xu J, Qian J, Xie X, Lin L, Zou Y, Fu M, Huang Z, Zhang G, Su Y, Ge J. High density lipoprotein protects mesenchymal stem cells from oxidative stress-induced apoptosis via activation of the pi3k/akt pathway and suppression of reactive oxygen species. Int J Mol Sci 2012; 13: 17104-17120.

[21] Muzi-Filho H, Bezerra CG, Souza AM, Boldrini LC, Takiya CM, Oliveira FL, Nesi RT, Valenca SS, Einicker-Lamas M, Vieyra A, Lara LS, Cunha VM. Undernutrition affects cell survival, oxidative stress, ca2+ handling and signaling pathways in vas deferens, crippling reproductive capacity. PLoS One 2013; 8: e69682.

[22] Fang SJ, Wu XS, Han ZH, Zhang XX, Wang CM, Li XY, Lu LQ, Zhang JL. Neuregulin-1 preconditioning protects the heart against ischemia/reperfusion injury through a pi3k/akt-dependent mechanism. Chin Med J (Engl) 2010; 123: 3597-3604.

[23] Ravingerova T, Matejikova J, Neckar J, Andelova E, Kolar F. Differential role of pi3k/akt pathway in the infarct size limitation and antiarrhythmic protection in the rat heart. Mol Cell Biochem 2007; 297: 111-120.

[24] Kim EK, Choi EJ. Pathological roles of mapk signaling pathways in human diseases. Biochim Biophys Acta 2010; 1802: 396-405.

[25] Eckardt K, Taube A, Eckel J. Obesity-associated insulin resistance in skeletal muscle: Role of lipid accumulation and physical inactivity. Rev Endocr Metab Disord 2011; 12: 163-172.

[26] Elliott B, Renshaw D, Getting S, Mackenzie R. The central role of myostatin in skeletal muscle and whole body homeostasis. Acta Physiol (Oxf) 2012; 205: 324-340.

[27] Qin Q, Niu J, Wang Z, Xu W, Qiao Z, Gu Y. Astragalus membranaceus inhibits inflammation via phospho-p38 mitogen-activated protein kinase (mapk) and nuclear factor (nf)-kappab pathways in advanced glycation end product-stimulated macrophages. Int J Mol Sci 2012; 13: 8379-8387.