Quest Journals
Journal of Research in Pharmaceutical Science
Volume 9 ~ Issue 5 (2023) pp: 01-14
ISSN(Online) : 2347-2995
www.questjournals.org

Research Paper

Berberine protects againstdoxorubicin-inducedcardiotoxicitybyrestraining oxidative stress and apoptosis associated with FOXO3a

JinboChen[a,b],SuwenSu[c], Feifei Mu[d], GuoqiangZhang[e], HuicaiGuo[af]*
[a]*Department of Toxicology, Hebei Medical University, Shijiazhuang, China*
[b]*Cardiovascular internal medicine, The Second Hospital of Shijiazhuang City, Shijiazhuang, China*
[c]*The Key Laboratory of Neural and Vascular Biology, Ministry of Education, Hebei Medical University, Shijiazhuang, China.*
[d]*Shijiazhuang medical college, Shijiazhuang, China*
[e]*Department of Dermatology, The First Hospital of Hebei Medical University, Shijiazhuang,China*
[f]*Hebei Key Laboratory of Environment and Human Health, Shijiazhuang, China*
Corresponding author: HuicaiGuo**,Department of Toxicology, School of Public Health, Hebei Medical University, 361 East Zhongshan Road, Shijiazhuang 050017, Hebei Province, China.*

Abstract
This study is aimed at investigating the protective effects of berberine (Ber) on Doxorubicin(DOX-)-triggered cardiac injuryand exploring whetherthe protective effect of Ber is related to themodulation of theForkhead box O3a (FOXO3a) pathway in mice and a humancardiomyocytes AC16 cell line. Our results showed Bersignificantly ameliorated the DOX-induced oxidative stress and apoptosis in vivo andin vitro. Importantly, treatment with Ber or DOX alone resulted in an increase of FOXO3a expression in mice heart and AC16 cells, co-treatment with Ber and DOX had a higher FOXO3aprotein level compared with DOX-only-treated group. After exposing AC16 cells to DOX, the increased FOXO3a expression induced by Ber and DOX was abrogated by the use of siRNA against FOXO3a. FOXO3a-deficient AC16 cells exhibited greater sensitivity to DOX-induced cell death, oxidative stress and apoptosis. Knockdown of FOXO3a markedly prevented the Berprereatment-induced rise in cell viabilityand the reduction of ROS generation and apoptosis. Exposure to Beror DOX alone increased the mRNA and protein expression of mitochondrial superoxide dismutase (Mn-SOD)andapoptosis repressor with caspase recruitment domain (ARC), more remarkable increases in the Mn-SOD and ARC levels were detected in theBer+DOX group. The upregulation of Mn-SOD and ARC were not observed in the FOXO3a-deficient AC16 cells. Taken together, our results indicated that FOXO3a-mediatedupregulation of Mn-SOD and ARC expression contributes to the preventionof DOX-induced cardiotoxicity,Ber may activate FOXO3a to upregulateMn-SOD and ARC and protect against DOX-induced cardiotoxicity.

Key words:*Berberine; doxorubicin; FOXO3a; oxidative stress; apoptosis; heart*

R*eceived 28 Apr., 2023;* ***R****evised 05May, 2023;* ***A****ccepted 07 May, 2023 © The author(s) 2023.*
P*ublished with open access at* ***www.questjournals.org***

I. Introduction

The doxorubicin (DOX) is one of the most prescribed and effective anti-cancerdrugs[1, 2]. However, the clinical use of DOX has been associated with a cumulative and dose-specific cardiotoxicity that involves the development of congestive heart failure[3, 4]. Multiple mechanisms are involved in DOX-induced cardiotoxicity,including an increase in reactive oxygen species (ROS), calcium overloading, and deteriorationof mitochondrial function, leading to impaired DNA andcardiomyocyte apoptosis [5-7]. Among them, oxidative stresshas been implicated as one of the major causes of DOXinduced cardiotoxicity.The search for an effective andsafe antagonist of the DOX-induced cardiac toxicity remains a critical issue in both cardiologyand oncology.Berberine (Ber), a natural compound, used in traditional chinese medicine. Among the many biological actions described for Ber, antioxidant is the most relevant[13-17]. Accumulating evidence has proved that Ber exhibitsremarkable beneficial effects in the cardiovascular system, providingprotection against

myocardial infarction [18,19], cardiac ischemia-reperfusion injury[20-22], heart failure[23], and DOX-induced cardiomyopathy[24,25].Wu et al have reported that Ber has a protective effect against DOXinduced cardiovascular injury through suppressing ROS production and cellularapoptosis[24]. Theexact mechanisms of this naturalproduct to impede the progression of DOX-induced cardiotoxicityrequire further study.

Forkhead box O3a (FOXO3a) (also named forkheadrhabdomyosarcoma-like 1, FOXO3a), a major member of the mammalian FOXO subfamily, plays an important role in metabolism, cell survival and resistance to oxidative stress and apoptosis in multiple cell types [8]. Recentstudies conducted by Zhu et al. demonstrated that FOXO3a is involved in the regulation ofDOX-induced cardiac toxicity. DOX induces a decreasedexpression of FOXO3a in cardiomyocytes. And enforced expression of FOXO3a inhibitsDOX-induced toxicity in cardiomyocytes[9], but the underlying mechanism has not beenfully elucidated. FOXO3a regulates detoxification of ROS through upregulation of mitochondrial superoxide dismutase (Mn-SOD)[10].The heart has evolutionarily developed a highly expressed apoptosis repressor with caspase recruitment domain (ARC)[11].FOXO3a is shown to inhibit apoptosis in cardiomyocytes through ARC, which is a direct transcriptional target of FOXO3a[12]. Whetherthe FOXO3a/Mn-SOD/ARC pathway is involved in DOX-induced cardiotoxicityis not clear.Activation by reagents such as resveratrol of FOXO3a has been shown to enhance cell resistance andsurvival from stress[26, 27]. Whether FOXO3asignalling participates in the protective effect of Ber in DOX-inducedcardiac dysfunction and its underlying mechanisms remainsundefined.

Taken together,the aims of the present study were to (1)examine the protective ability of Ber against DOXinduced cardiotoxicity by regulating ROS generationand apoptosis; (2) determine whethertheFOXO3a/Mn-SOD/ARC pathway is involved in DOX-induced cardiotoxicity in mice and AC16 human cardiomyocytes; and (3) determine whetherthe regulating protective effect of Ber is mainly related to themodulation of theFOXO3a/Mn-SOD/ARCpathway.

II. Materials And Methods

2.1. Reagents

DOX was purchased from AccuStandard (AccuStandard, Inc., New Haven, CT, USA). Ber was provided by AcrosOrganics,Belgium. Dimethyl sulfoxide (DMSO) was purchased from Solarbio (Solarbio Science & Technology Co., Ltd., Beijing, China), Culture media (DMEM/F12) and fetal bovine serum (FBS) were fromGibco(Invitrogen Corporation, New York, USA). FITC-labeled annexin V (AnnexinV-FITC) and propidium iodide (PI) were obtained from apoptosis detection kit (Ann Arbor, Inc., MI, USA). All other chemicals were either analytical or high pressure liquid chromatography (HPLC) grade.

2.2.Animals and Treatments

Kunming mice weighing18-22g were obtained from the medical laboratory ofHebei Medical University. All animals were housed in thenew environment for one week in a standard experimentalroom (12 h light/dark cycles) with free access to tap water. This study conformed to the Guide for the Care and Use ofLaboratory Animals (NIH Publication No. 85-23, Revised2016). All experimental procedures performed in the currentstudy followed the guidelines of the research ethics committee of Hebei Medical University (Shijiazhuang, China). In thecurrent study, forty mice were randomly assigned to four groups (saline, DOX, Ber and Ber + DOX) and treated as follows. From day 1 to day 10, animals of Bergroup and Ber + DOX group were orally administered with Ber at the dose of 25 mg /kg (once a day for 10 days), while animals of saline group and DOX group were administered with equal volume of physiological saline. On day 7, a single dose of DOX (20 mg/kg, i.p.) was administered to animals of DOX group and Ber + DOX group, while equal volume of physiological saline was administered to animals of saline group and DOX group. Animals were sacrificed on the third day after DOX injection. Blood was collected and hearts were removed for further determinations as described below.

2.3. Serum biochemical assays Blood sample was collected from the inner canthus of each mouse prior to sacrifice. The blood was centrifuged at 3000 g for 15 min to separate the sera, which were stored at -80°C for the biochemical analyses. Creatine phosphokinase (CK) and creatine kinase isoenzymeMB (CK-MB) activities were determined according to standard methods using diagnostic kits from BioSystems S.A. (Barcelona, Spain) and a CHEMIX-180 automatic biochemistry analyzer (Sysmex).

2.4. Histopathological examination of the heart tissue

Paraformaldehyde-fixed heart tissue samples were embedded in paraffin wax, seriallysectioned (5 μm thickness) and stained withhematoxylin and eosin for the assessment ofthe histopathological changes.

2.5.Apoptosis assessment using TUNEL assay

The TUNEL (terminal deoxynucleotidetransferase-mediated dUTP nick end-labelling) stainingwas performed using an in situ cell apoptosisdetection kit (Boster Biological Technology, Wuhan, Hubei, China). According to our previous study[28], paraffin-embedded sections of samples were deparaffinized and hydrated, and then incubated in 20 g/ml protease K at room temperature for 5 min. Afterbeing washed twice, the samples were

transferred to sodium citrate buffer (2 mmol/L citricacid and 10 mmol/L trisodium citrate, pH 6.0)at 37.8°C for 5 min. After two more 5-min washes with phosphate-buffered saline (PBS), thesamples were transferred to 20 ml of the TUNELreaction mixture (1 ml terminal deoxynucleotidyltransferase, 1 ml digoxin-labelled d-UTPand 18 ml Labelling Buffer) and incubated at37.8°C for 60 min. After rinsing, the sectionswere incubated with a biotinylated anti-digoxinantibody for 30 min at 37.8°C and developedwith DAB substrate kit. The slides were lightlycounterstained with hematoxylin and then dehydrated and mounted. For each myocardialspecimen, the tissue sections were examinedmicroscopically at × 400 magnification, and 10random fields per section were counted. Thepercentage of apoptotic cells was calculated asthe apoptotic index, i.e., the ratio of the numberof positively stained myocyte nuclei to the totalnumber of myocyte nuclei.

2.6. Cell culture and chemical exposures

Human cardiomyocytes AC16 cell line were derived from America Tissue Type Collection (ATCC, Bethesda, MD, USA). Cardiomyocytes were grown in DMEM/F12 with 10% FBS, penicillin–streptomycin (100 μg/ml, respectively). Cultures were incubated in an atmosphere of 5% CO_2 at a temperature of 37 °C. The Cells were treated with DOX at concentrations of 1, 2, 4, 8μM for 24 h to investigate DOX induced toxicity.The Cells were treated with Ber at concentrations of 0.1, 0.5, or 1 μM for 4 h and then exposed to 2μMDOX for 24 h.Treated and untreated cells at the indicated time points were harvested for analysis.

2.7. Cell viability

The viability of cardiomyocytes AC16 was determined by MTT assay. Briefly, 5×10^3 cells were seeded in 96-well plates and were maintained in culture media for 24 h, followed by exposure with the indicated concentrations of Ber or DOX. The negative control and blank control groups were treated with < 0.1% DMSO and the medium, respectively. After exposure, 50 μl MTT dye solution (1 mg/ml in PBS) was added to each well containing 150 μl medium. After 4 h incubation at 37 °C, formazan crystals were dissolved with 150 μl DMSO per well and the absorbance was measured on a microplate reader (Prelong corporation, Beijing, China) at 495 nm. Each exposure group consisted of six replicate wells. The percentage of cell viability was calculated according to the following formula: cell survival (%) = (A495 treated - A495 blank)/ (A495 negative - A495 blank) × 100%. Results were expressed as percentage of corresponding control.

2.8. Intracellular ROS measurement

The intracellular ROS generation was detected by Flow cytometry using 2,7-dichlorofluorescein diacetate (DCFH-DA) as a probe.According to our previous study[29], the cell suspension was isolatedfrom heart. Briefly, cell suspension and AC16 cells were incubated with DCFH-DA (Bryotime Institute of Biotechnology, China) at a final concentration of 5 μM. The formation of the fluorescent-oxidized derivative of DCF was monitored using a BD AccuriC6 flow cytometer (Becton Dickinson, USA) at emission wavelength of 525 nm and excitation wavelength of 488 nm. Finally, ROS generation was quantified by the median fluorescence intensity of 10,000 cells.

2.9. Apoptosis assay by flow cytometry

The frequencies of apoptosis were determined by AnnexinV-FITC/propidium iodide (PI) double staining method. The cells were collected and incubated in 500 μl binding buffer containing 10 μl PI and 5 μlAnnexinV in the dark at room temperature for 15 min. Then the stained cells were analyzed by a BD AccuriC6 flow cytometer (Becton Dickinson, USA). Data of 5×10^4 cells per sample were collected and analyzed in each experiment.

2.10. Western blotting

The hearts in mice were homogenized in RIPA bufferusing a high throughput tissuegrinder (Scientz, Ningbo, China). Whole cell proteinfrom AC16 cells were extracted with RIPA buffer. The proteins concentration was determined by a bicinchoninic acid protein assay (Beyotime Biotech, Beijing, China). An equal amount of protein (50 μg) from each sample was resolved by SDS-PAGE, transferred to PVDF membranes and blocked with 5% nonfat milk in TBST (50 mMTris-HCl, 150 mMNaCl, 0.1% Tween, pH 7.4) for 1 h at room temperature. Membranes were then incubated with the polyclonal IgG for FoxO3a (Abcam Inc., Cambridge,UK, 1:3000 diluted with 1× TBST),Mn-SOD and β-actin (KPL Scaffold Inc, California, USA，1:2000 diluted with 1× TBST) and ARC (Affinity BioReagents, dilution 1:200) over night at 4°C, washed with TBST three times for 10 min each, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:2000) for 1 h at 37°C. After washing, blots were detected using an enhanced chemiluminescenceplus system (ZhongShan Bioengineering Institute, Beijing, China). Protein bands of the blots were observed and taken pictures using automatic chemiluminescence image analysis system (Tanon Science & Technology Co., Ltd, Shanghai, China) and then analyzed with the gel-pro32 software (Roper Technologies, Inc., Sarasota, USA). The

data of the target proteins were normalized to the ratios of β-actin detected on the same blot to control for possible variations in protein loading.

2.11. Quantitative real-time polymerase chain reaction

Total RNA was extracted by Trizol (Invitrogen Co., Carlsbad, USA) and isolated RNA was quantified by Nano-100 microspectrophotometer (Allsheng instrument Co., Ltd, Hangzhou, China). RNA was reverse-transcribed to cDNA using a PrimeScript RT reagent kit with a gDNA Eraser cDNA synthesis kit according to the manufacturer's protocol (Applied Biological Materials Inc, Richmond, Canada). The results were normalized against the expression of endogenous control β-actin. Each reaction was performed in triplicate. A quantitative real-time polymerase chain reaction (qRT-PCR) analysis was performed using a FQD-96A real time-PCR instrument (Bioer Technology Co., Ltd, Hangzhou, China) and a KAPA SYBR FAST qPCR Kit (Applied Biological Materials Inc, Richmond, Canada). Relative quantification of each gene expression was calculated according to the $2^{-\Delta\Delta CT}$ methodology using the Biorad software tool Genex-Gene Expression MacroTM. All the primers were obtained from Sangon, China. Primers sequences were as follows:

Gene	Sense (5′ →3′)	Anti-sense (3′ →5′)
FOXO3a	CTTCAAGGATAAGGGCGACA	TCTTGCCAGTTCCCTCATT
Mn-SOD	AATGGAGGTCAGAGTTTAG	AATAGCCAGGGAAGTTAG
ARC	ATCCAGTCCTCAGCCCTAAT	GCTCTAAGCCCTCCCTATGT
β-actin	CGGGAAATCGTGCGTGACAT	GAACTTTGGGGGATGCTCGC

2.12.RNA Interference

The FOXO3asiRNA sequences were as follows: sense 5′-CCCUGUAAAGCUUUCAGAAdtdt-3′and antisense 5′–UUCUGAAAGCUUUACAGGGdtdt-3′. The FOXO3a -specific siRNA and control siRNA were obtained from Santa Cruz Biotechnology Co. (Santa Cruz, CA, USA), and transient siRNA transfection was carried out according to ourprevious study[30].AC16 cells were plated in six-well plates at a density of 2×10^5 cells per well in 2 ml DMEM/F12. When the cells reached 60–70% confluence the next day, transient transfection was carried out using Lipofectamine 2000 according to the manufacturer's instructions.In brief, 1 μlsiRNA (5 μMsolution) and 3 μlLipofectamine 2000 were diluted separately with 100 μL DMEM/F12 reduced serum medium and kept at room temperature for 5 min. The diluted siRNA and Lipofectamine 2000 were mixed gently followed by incubation for 20 min at room temperature. 200 μl of siRNA-Lipofectamine complex was added to each well containing 800 μl DMEM/F12 without antibiotics. After a 5 h incubation of cells with siRNA-Lipofectamine 2000 complex, the medium was replaced with normal DMEM/F12, and the cells were maintained for an additional 24 h. FOXO3a knockdown were determined by qRT-PCR and Western blot analysis.

2.13. Statistics

All statistical analyses were conducted with SPSS Statistics 21 (SPSS Software, IBM). Multiplecomparisons were performed using a one-way analysis ofvariance (ANOVA), followed by Bonferroni multiple comparison post hoc tests. Comparisons between two groupswere performed by using Student's *t*-test. Statistical significance was set to $p < 0.05$ and the data were represented as mean ± SD.

III. Results

3.1. Ber reduced DOX-induced cardiotoxicity in mice and AC16cardiomyocytes

We first explored whether Ber could protectmice from acute DOX-induced cardiac injury.Light microscopic examination of heart sections after 72 h of DOX treatment revealedvascular congestion, oedema of myocardial tissue and cells and lossof striation (Fig. 1A). Incontrast, DOX-induced histopathological changes were attenuatedpartially in the Ber+DOX group.The serum markers indicating myocardial injury, CK andCK-MB, were significantly elevated in the DOX-only-treated group compared with the control group. Pretreatment with Ber significantly reduced the CK andCK-MB levels compared with the DOX group(Fig.1B, C). Next, the cytoprotectiveeffect of Ber was then examined in vitro using an AC16humancardiomyocytecell model.To determine the cell viability following treatment with different concentrations of DOX and Berin the cell line, an MTT assay was performed. The exposureof the AC16 cells to DOX for 24 h resulted in a significantreduction in cell viability in a dose-dependent manner(Fig 2A). As shown in Fig. 2B,Ber (0.1μM,1μM, 10μM) showed no additional benefit to cell viability.DOX（2μM）significantly reduced cell viability to approximately55% of the control levels, whereas Ber showed a protective effectagainst DOX-induced cytotoxicitycompared with DOX-treated cells ($p < 0.05$).

Fig. 1 Effects of Ber on histopathological changes and myocardial enzyme level in DOX treated cardiac tissue. (A) Effects of Ber on histopathological changes. Con:normalcardiac tissue; Ber: cardiac tissue treated with Ber (25 mg/kg); DOX: DOX treated cardiac tissue(20 mg/kg);DOX+Ber: DOX treated cardiac tissue with Ber. ↑vascular congestion;: oedema of myocardial tissue and cells.(H&E 400×).(B)and (C) Effects of Ber on myocardial enzyme level.Each column represents the mean ± SD, n = 6.*p< 0.05 vs. control; #p< 0.05 vs. DOX.

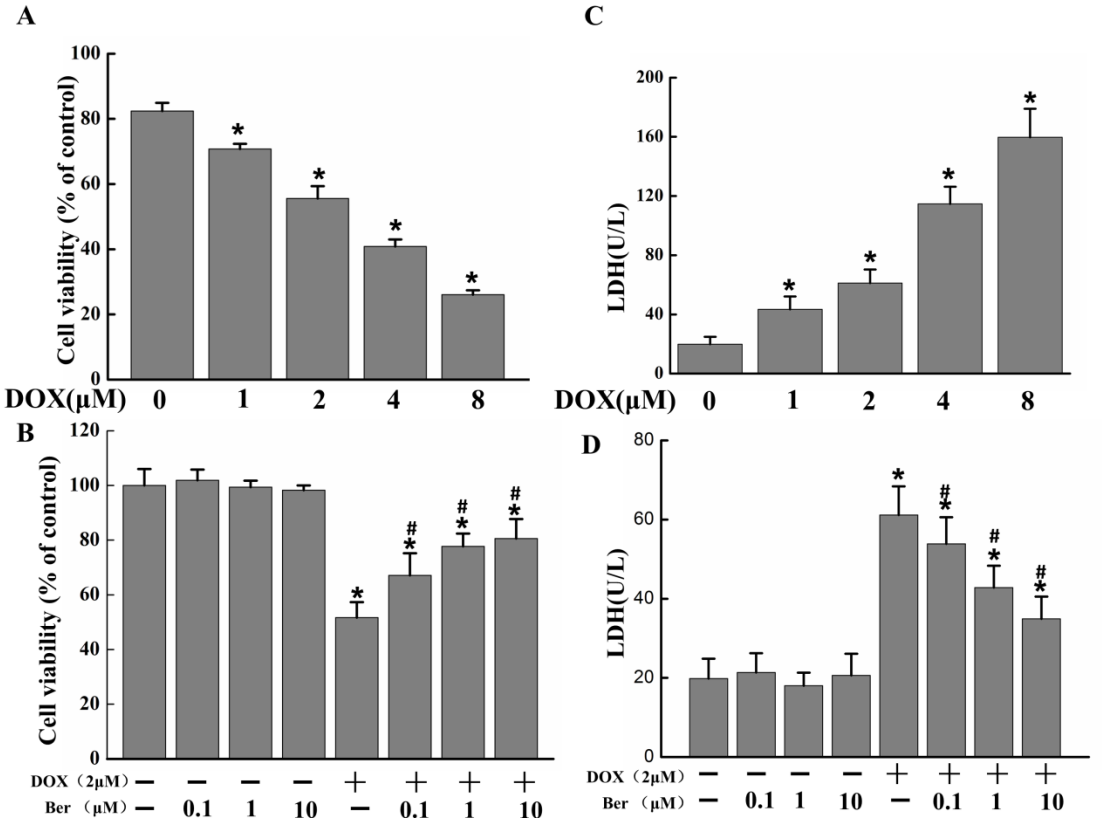

Fig. 2 Impact of DOX and Ber on cell viability ofAC16 cells.(A) Dose-dependent effect of DOXoncell viability，The Cells were treated with DOX at concentrations of 1, 2, 4, 8μM for 24 h;(B) Effects of Ber on the reduction in the viability rate induced by DOX，The Cells were treated with Ber at concentrations of 0.1, 1, or 10 μM for 24 h and then exposed to 2μMDOX for 24 h. Each column represents the mean ± SD,n = 6.*p< 0.05 vs. control; #p< 0.05 vs. DOX.

3.2.Ber ameliorated DOX-induced oxidative stressandapoptosisin vivo andin vitro

On the basis that oxidative stress is consideredto be the primary cause of DOX-induced cardiomyopathy，andBer is known to protect cells from oxidative stress. We further evaluated the myocardialoxidative stress induced by DOX. Compared with the control group, ROS levels increased 2.37-fold in heartof mice after treatmentwith DOX. Pretreatment with 25 mg/kg Ber prevented nearly 85%of theincrease of the ROS levels in heart(Fig. 3C).Treatment with 2μM DOX for 24 h caused a2.32-fold increase in DCF fluorescence in AC16 cells, suggesting theintracellular production of ROS. Pretreatment with 1μMBer for 24 h dramatically reduced DOX-induced freeradical release (Fig. 4C).Because oxidative stress can induce apoptosisand myocardial apoptosis is also a causativefactor in cardiomyopathy, we examined theapoptotic changes using the TUNEL assay. Asshown in Figure 3A and B, the apoptotic index (TUNELpositivecells) following DOX treatment was significantlyincreased from5.28% to18.14%, but the apoptotic index was decreased to11.84% by the combined treatment withBer.Additionally, apoptotic cell death by DOX with and withoutBerpretreatment was also quantified using Annexin V-FITC staining assay inAC16 cells. Fig. 4A and B shows that treatment with 2μM DOX for 24 h increased the percentage of apoptotic cells,whereas pretreatmentwith 1 μMBer reducedapoptotic cell death (1.72 in control, 19.4 in DOX treated, 10.97 incotreated).

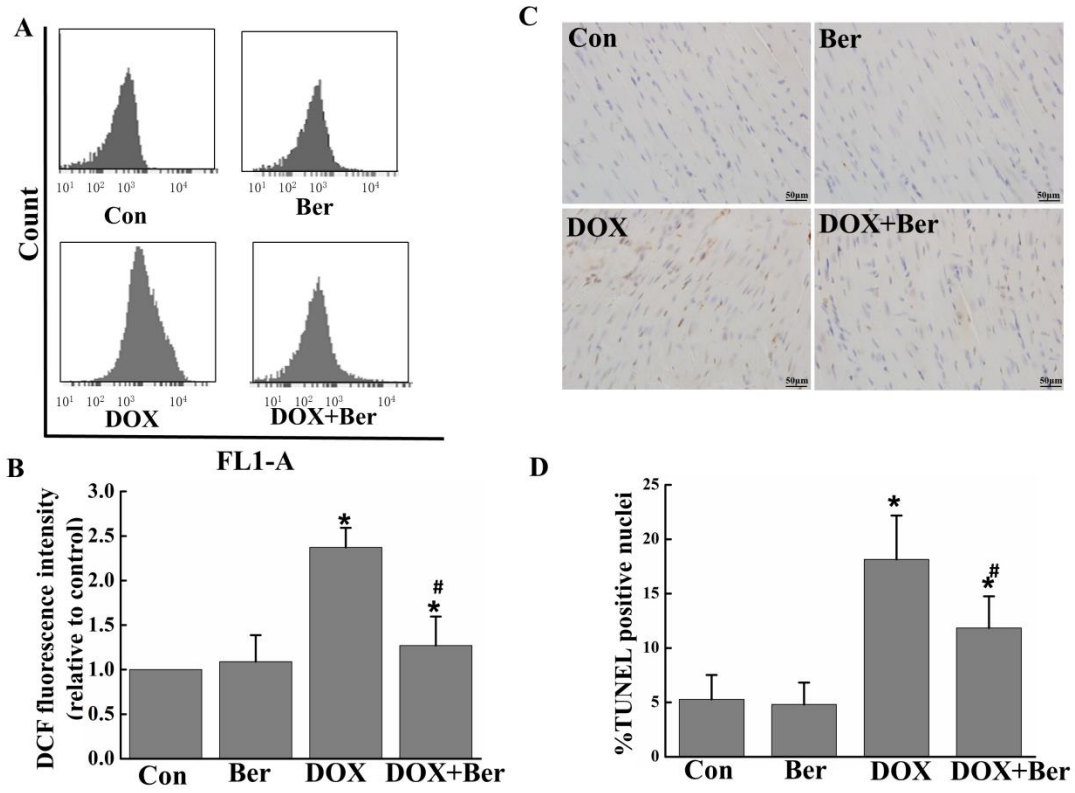

Fig. 3Effects of DOX and Ber on the ROS production and apoptosisin mice heart.(A)and(B)Apoptotic myocytes in themouse myocardia were detected byTUNEL staining. TUNEL-positive nucleiare indicated by brown staining. (C) Intracellular ROS generation measured by flowcytometry.Each column represents the mean ± SD, n = 6.*p< 0.05 vs. control; #p< 0.05 vs. DOX.

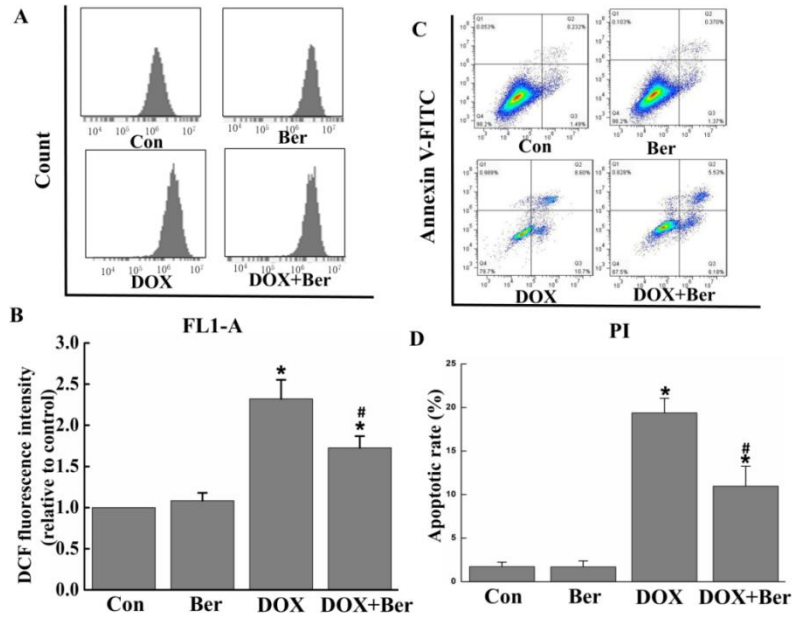

Fig. 4Effects of DOX and Ber on the ROS production and apoptosisin AC16 cells.(A)and(B)Apoptosiswasanalysed by flow cytometry with FITC-conjugated Annexin V/propidium iodide.(C) Intracellular ROS generation measured by flowcytometry.n = 6.*p< 0.05 vs. corresponding control; #p< 0.05 vs. DOX.

3.3.Ber and DOXupregulated FOXO3a expression in mice heart and AC16 cells

FOXO3a has the ability to resist to oxidative stress and apoptosis.To understand whetherFOXO3a is involved in Ber-mediated protection against DOX induced toxicity,we treated mice and AC16 cells with DOX or Ber and examined the FOXO3a levels.Ber increased FOXO3a mRNA levelin mice heart and AC16 cellscompared with control, however, DOX had no effect on FOXO3a mRNA level, DOX+Ber group had a more higher FOXO3a mRNA level compared with DOX-only-treated group(Fig 5A, C). Western blot assay confirmed thatBer also upregulated total FOXO3a protein expressioncompared with control. Further, our results showed that treatment with DOX alone resulted in an increase of FOXO3a protein expression in mice heart and AC16 cells,DOX +Ber group had a more higher FOXO3aprotein level compared with DOX-only-treated group(Fig 5B, D).

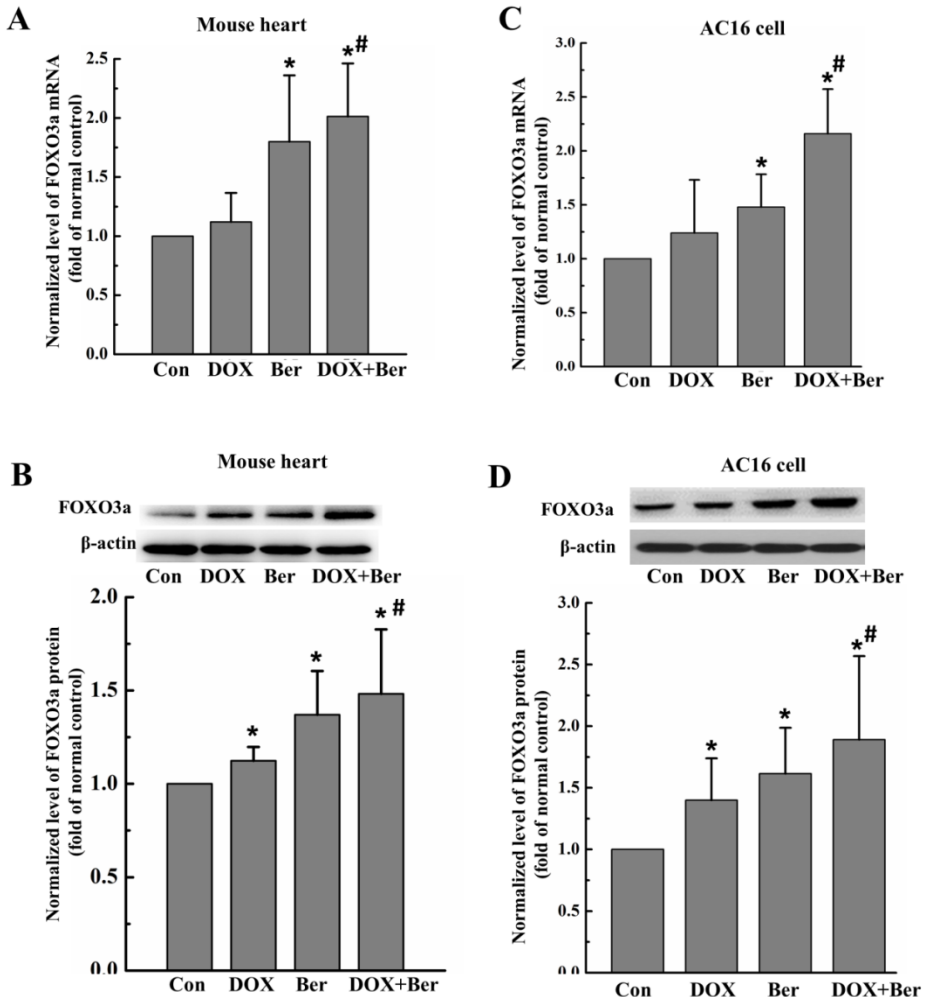

Fig. 5 Effect of DOX on the expression of FOXO3a in mice heart and AC16 cardiomyocytes. (A)and(B)The mRNA and protein expression of FOXO3a in mice heartwasdetermined.Each bar represents mean ± SD, n=5 per group (**p*< 0.05,#*P*< 0.05 vs. DOX).(C)and(D)The mRNA and protein expression of FOXO3a in AC16 cellswasdetermined.Each column represents the mean ± SD, n = 6.**p*< 0.05 vs. control; #*p*< 0.05 vs. DOX.

3.4.FOXO3a plays an essential role in Ber-mediated protection against DOX induced toxicity

To study the role of FOXO3a in Ber-mediated protectionagainst DOX-induced toxicity, we transiently knocked down FOXO3a inAC16 cells by siRNA oligonucleotides. RT-PCR and Western blot analyses showed that FOXO3asiRNA efficiently knocked down FOXO3a expression in AC16 cells.Silencing against FOXO3a suppressed Ber or DOX-induced FOXO3a expression in AC16 cells (Fig. 6).FOXO3a-deficient AC16 cells exhibited greater sensitivity to DOX-induced cell death, oxidative stress and apoptosis compared to the cells transfected with siRNA-control (Fig. 7). In DOX-treated AC16 cells, knockdown of FOXO3a markedly prevented the Berpretreatment-induced rise in cell viabilityand the reduction of ROS generation and apoptosis

rate.Taken together, inhibition of FOXO3a activation reversed Ber-inducedprotection against DOX cardiotoxicity.

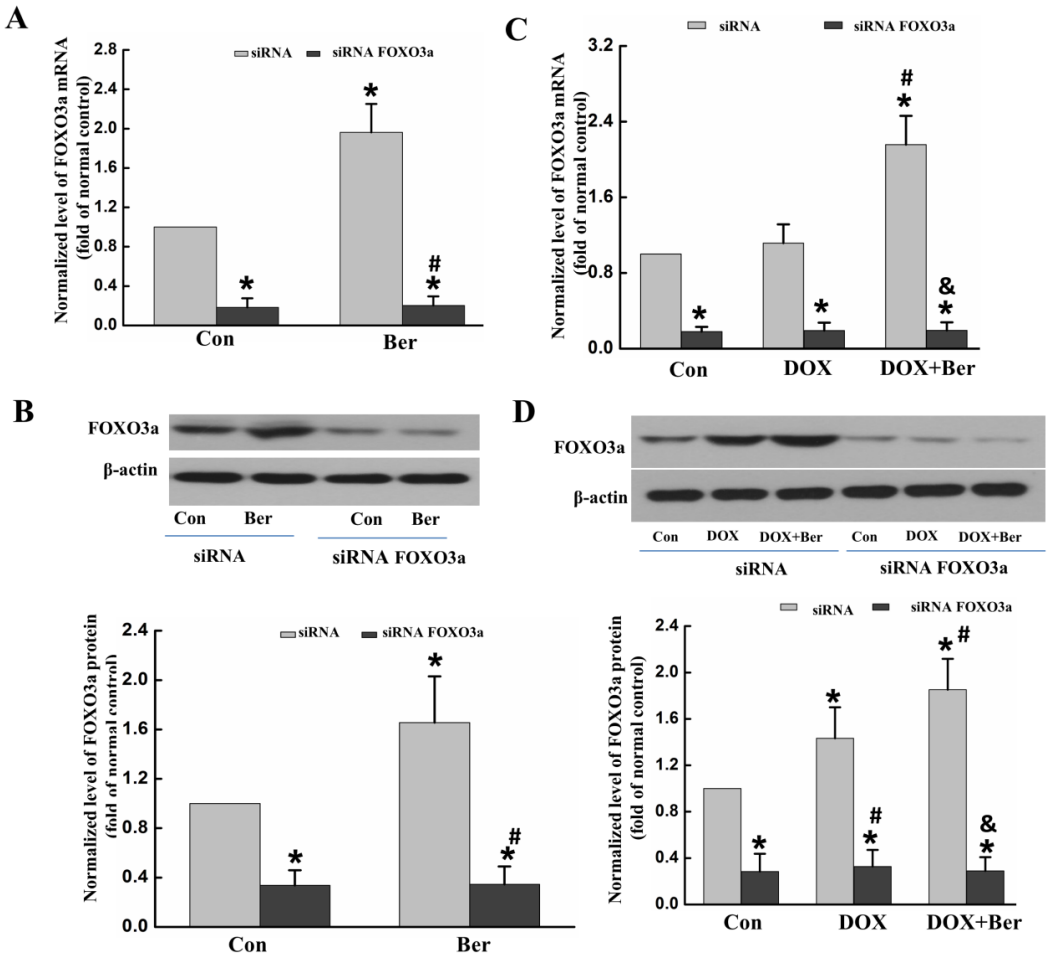

Fig. 6 Effect ofFOXOa deficiencyon the expression of FOXO3a in AC16 cardiomyocytes. (A)and (C) FOXOa deficiencyantagonisetheupregulation of mRNAexpression of FOXO3a induced by Ber and DOX.(B) and (D) FOXOa deficiencyantagonisetheupregulation of protein expression of FOXO3a induced by Ber and DOX. Each bar represents mean ± SD,n = 6.*p< 0.05 vs. siRNAcontrol; $^{\#}p$< 0.05 vs. siRNADOX;$^{\&}p$< 0.05 vs. siRNADOX+Ber.

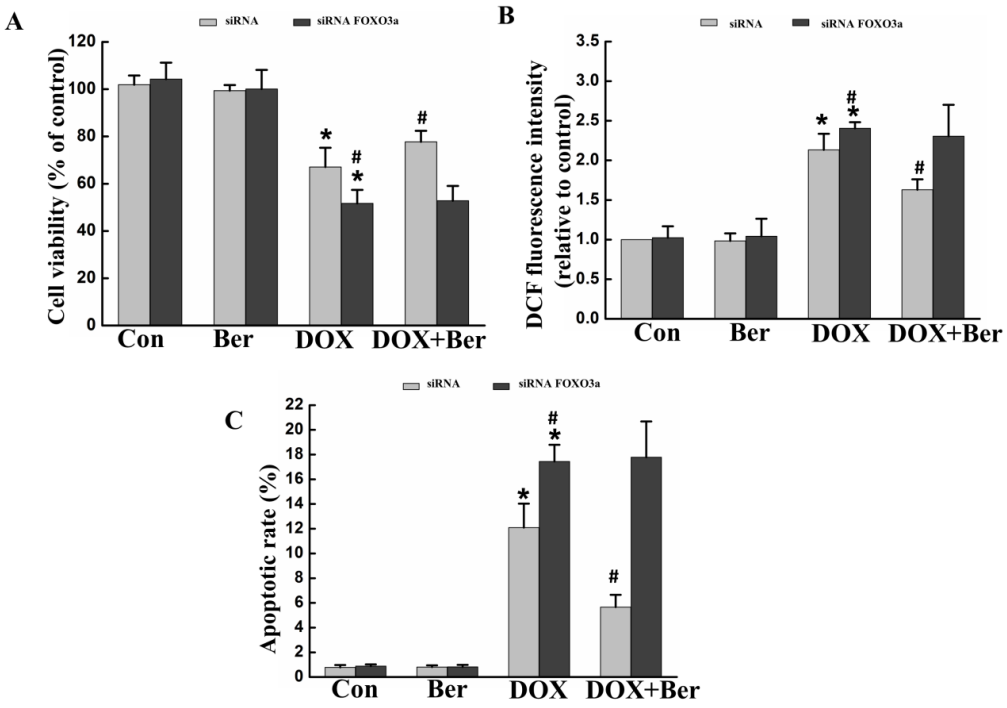

Fig. 7Effect ofFOXOa deficiency on the DOX-induced cytotoxicity,ROS production and apoptosis in AC16 cardiomyocytes.AC16cardiomyocytes were exposed tocontrol siRNA or FOXO3asiRNA and then treated with Ber (1 μM for 24 h). Then, the cells were exposed to 2μM DOX for a 24 htreatment.(A)FOXO3a deficiency aggravated DOX-induced cytotoxicityandantagonise the protectiveeffect of Ber on cell viability.(B) FOXO3a deficiency aggravated DOX-induced ROS productionandantagonise the protectiveeffect of Ber on ROS release.(C) FOXO3a deficiency aggravated DOX-induced apoptosisandantagonise the protectiveeffect of Ber on apoptosis.n = 6.*p< 0.05 vs. siRNAcontrol; $^{\#}p$< 0.05 vs. siRNA DOX.

3.5.Berupregulated the expression of Mn-SOD and ARC in AC16 cardiac cells

We further examined the FOXO3a transcriptional activity by measuring the expression of its downstream anti-oxidative and anti-apoptosis genes. Exposure to Ber alone increased the mRNA and protein expression of Mn-SOD and ARC.However, the upregulation of Mn-SOD and ARC were not observed in the FOXO3a-deficient AC16 cells (Fig.8).Treatment withDOX alone also increased the mRNA and protein expression of Mn-SOD and ARC, more remarkable increases in the Mn-SOD and ARC levels were detected in theBer+DOX group. Similarly, the upregulation of Mn-SOD and ARC were not observed in the FOXO3a-deficient AC16 cells. These results suggested that the upregulation of Mn-SOD and ARC byBer or DOX was FOXO3a-dependent. Therefore, the inability of Ber to alleviate the DOX-induced toxicity in the FOXO3a-deficient AC16 cells is probably attributable to the loss of the FOXO3a-dependent defensive response.

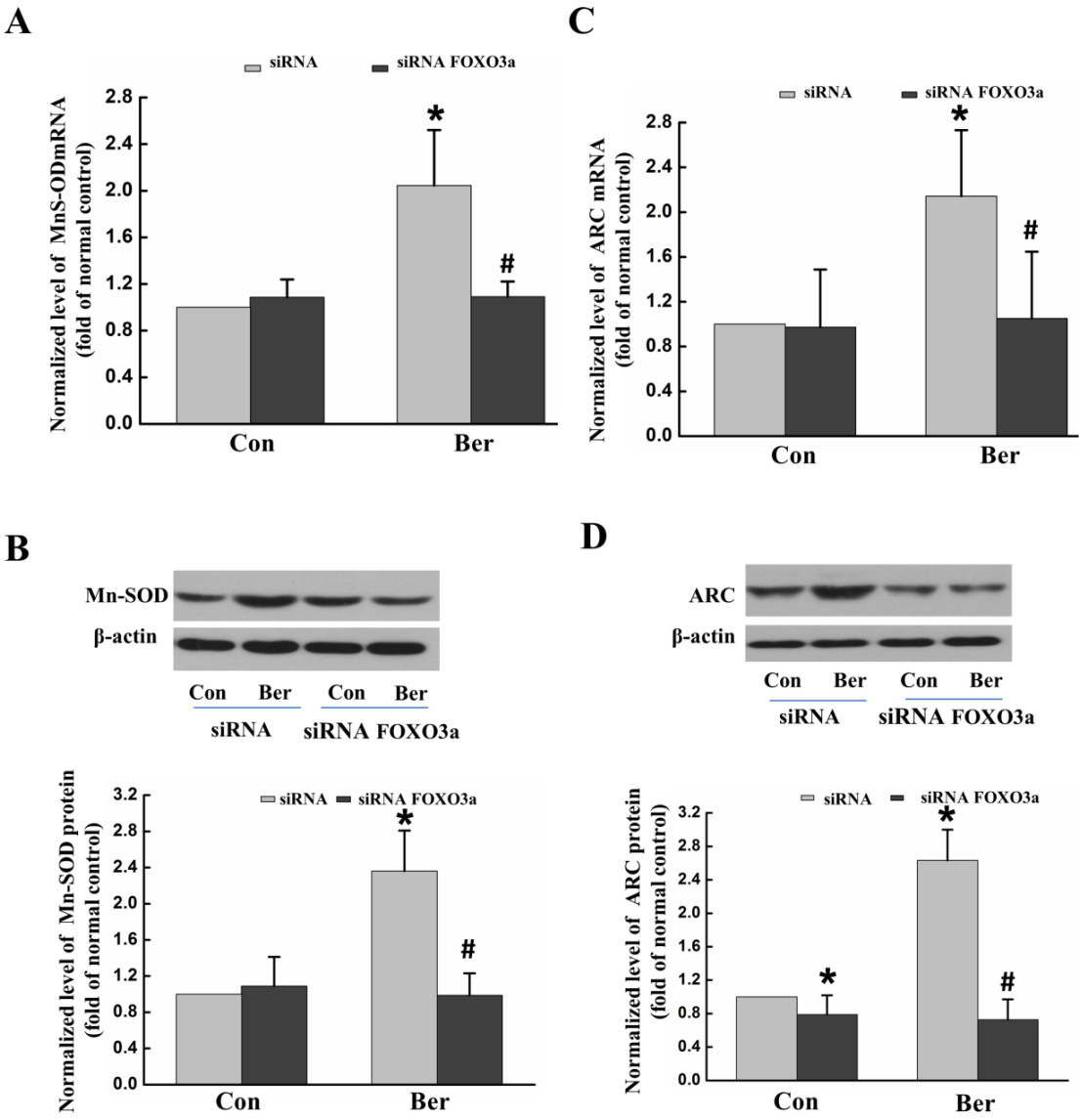

Fig. 8 Effect of Ber on the expression of MnSOD and ARC in AC16 cardiomyocytes. (A)and (C) FOXO3a deficiencyantagonizethe upregulation of mRNAexpression of MnSOD and ARC induced by Ber. (B) and (D) FOXOa deficiencyantagonizethe upregulation of protein expression of MnSOD and ARC induced by Ber. Each bar represents mean ± SD, n = 6.*p< 0.05 vs. siRNAcontrol; $^{\#}p$< 0.05 vs. siRNADOX.

IV. Disscussion

Berberine (Ber) is an alkaloid extract from the Coptischinensis species. Ber has a long history of use for the treatmentof diarrhoea in oriental medicine [39]. Increasing studieshave revealed that Ber is an effective antioxidant and free radical scavenger that possesses a variety of pharmacological andbiological activities[40]. Attractively, investigationshave shown that Ber exerts anticancer activity and can be apotential multispectrum anticancer agent [41- 43], indicatingthat a treatment combining Ber with DOX does not interferewith the antitumour effect of DOX and significantly inhibitscancer cell proliferation[44, 45]. In the present study, wedemonstrated that Ber treatment exhibits a significantprotective effect on cardiac tissue in animal and in vitro cellculture studies of DOX-induced cardiac injury. The Ber therapy significantly attenuated the histopathological deteriorations and reduced the CK andCK-MB levels. In parallelwith this effect, Ber could protect DOX-induced AC16cardiomyocytes by improving the cell survival ability caused by DOX. Further, Ber treatment significantlyalleviated the DOX-induced oxidative stress and apoptosis in vivo and in vitro.

FOXO3a is a transcription factor that belongs to the Forkhead family, which hasa DNA binding domain, called "Forkhead box". FOXO3a participates in the regulationof the cell cycle, differentiation, tumorigenesis, protection against oxidative stressand apoptosis[33]. FOXO3a is an important regulator in cardiomyocytes.The previouswork shows that FOXO3a is involved in the inhibition of cardiomyocyte hypertrophyand it promotes

cellular growth in cardiac remodeling[34].FOXO3a has beenrecently shown to be active and play important roles in DOX-inducedepithelial-mesenchymal transition in HCC cells[35].Increased nuclearexpression of FOXO3a is detected in H9C2 cells after DOX treatment[36].Our presentresults found thatDOX treatmentincreased the protein expression levels of FOXO3a (Fig. 5B and D).Further, we found that the absence of FOXO3a increases DOX-induced oxidative stress and apoptosis(Fig.8B and C). It was reasonable to conclude that FOXO3a activation confers protection against DOX-induced oxidative stress and apoptosis in AC16 cardiomyocytes. These findings are consistentwith previous studies that increase in the expression of FOXO3a reduces ROS and promotes cardiomyocytesurvival[37]. Zhu et al., however,illustrated that FOXO3a was down-regulated in the mouse heart in response to DOX[9].This contradiction may be related to the different time and dose of administration, but on the whole, FOXO3a activation can alleviate the cardiotoxicity caused by DOX.Hence, the regulation of FOXO3a expression might bea promising therapeutic approach to conquer DOX cardiotoxicity.It is particularly important to identify and develop FOXO3a activators.

Our data also showed that DOX induced a marked increase in the Mn-SOD and ARC level,which are the target genes of FOXO3a.Further, silencing FOXO3a markedly attenuated this increase in Mn-SOD and ARC induced by DOX. Mn-SOD is thought to play an important role in cellular defense against oxidative damage caused by ROS[38]. FOXO3a regulates detoxification of ROS through upregulation of Mn-SOD[10]. The heart has evolutionarily developed a highly expressed anti-apoptotic protein, ARC [11],which is a direct transcriptional target of FOXO3a[12].Thesefindings revealed that FOXO3a can transactivateMn-SOD and ARC. Thus, increased expression of FOXO3a contributes to Mn-SOD and ARC up-regulation, which attenuates DOX-induced oxidative stress and apoptosis in cardiomyocytes. We provide novel evidence that FOXO3a, Mn-SOD, and ARC constitute an anti-oxidative and anti-apoptotic pathway that participates in protection againstDOX-induced toxicity incardiomyocytes.And FOXO3a/Mn-SOD/ARCpathwaymay be promising therapeutic targets toenhance cancer therapy and cardioprotection simultaneously.

To elucidate the potential protective effects ofBer againstDOX-induced cardiotoxicity as well as the mechanismsresponsible for these effects, we observed the effect of Beron the FOXO3a expression inducedby DOX exposure. Our results showed that treatment with 1 μMBer alone resulted in an increase of FOXO3a expression (Fig. 8A). Pre-treatment with 10 μMBer followed by 2μM DOX resulted in a further increase inFOXO3a expression when compared with DOX group, and this was accompanied by an increase incell viability, indicating that theFOXO3a pathwaymay be involved in the protective effects of Beragainst DOX-induced cardiotoxicity.SilencingFOXO3a markedly prevented the Bercotreatment-induced rise in cell viability.These results indicate that Berinhibited DOX-induced toxicity in a FOXO3a-dependent manner.Exposure to Ber or DOX alone increased the mRNA and protein expression of Mn-SOD and ARC, more remarkable increases in the Mn-SOD and ARC levels were detected in theBer+DOX group. However, the upregulation of Mn-SOD and ARC were not observed in the FOXO3a-deficient AC16 cells.These findingssuggest that the Ber treatment led to FOXO3aupregulation,indicating that the protective effects of Ber against DOXinduced injury maybe related to the FOXO3a-mediated upregulation of Mn-SOD and ARC expression.

In conclusion, FOXO3a-mediatedupregulation of Mn-SOD and ARC expression contributes to the preventionof DOX-induced cardiotoxicity, suggesting that theFOXO3a pathway may represent an attractive target.Ber has a protective effect against DOX-induced cardiovascular injury, which is correlated with the activation of FOXO3a and the upregulation of Mn-SOD and ARCexpression, resulting in suppressed ROS production and apoptosis to improve cardiacdysfunction. This natural product should be developed as anew potential candidate to prevent or reduce the cardiac sideeffects of anthracyclines in chemotherapy. Of course, theexact mechanisms and clinical applications of this naturalproduct to impede the progression ofDOX-induced cardiotoxicity require further study.

V. Conclusion

Our in vivo and in vitro results showed that Ber preconditioningafforded protection against DOX-induced cardiotoxicityby reducing oxidative stress and apoptosis. Mechanistically, the protective effect involvestheFOXO3a/Mn-SOD/ARCpathway, suggesting thatthis pathway is an novel potential therapeutic target fordecreasing DOX toxicity.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Funding Statement

The present study was financially supported by the NaturalScience Foundation of China (grant no.21976050) andthe Shijiazhuang Science and Technology Research and development programme(grant no.191460933), andthe Project of Medical Science Research of Hebei Health Commission(grant no.20181093).

Reference

[1]. C. Carvalho, R.X. Santos, S. Cardoso, et al. Doxorubicin: the good, the bad and the ugly effect.Curr Med Chem 2009; 16: 3267-3285.
[2]. I Wolf, S. Sadetzki, I Gluck, et al.Association between diabetes mellitus and adversecharacteristics of breast cancer at presentation.Eur J Cancer 2003; 42: 1077-1082.
[3]. K. Chatterjee, J. Zhang, N. Honbo, et al. Doxorubicin cardiomyopathy. Cardiology2010; 11: 155-162.
[4]. Y. Octavia, C.G. Tocchetti, K.L. Gabrielson, S. Janssens, et al. Doxorubicininducedcardiomyopathy: from molecular mechanismsto therapeutic strategies. J MolCellCardiol 2012; 52: 1213-1225
[5]. E. Adameova, A.Goncalvesova,N.S.Szobi, etal.Necroptotic cell death infailing heart: relevance and proposed mechanisms, Heart Fail. Rev. 21 (2016)213 – 221.
[6]. Q. Guo, J. Guo, R. Yang, et al.CyclovirobuxineDattenuates doxorubicin-induced cardiomyopathy by suppression of oxidative damageand mitochondrial biogenesis impairment. Oxid. Med. Cell. Longev. 2015, 151972.
[7]. P. Bai, J.G.Mabley, L.Liaudet, et al.Matrixmetalloproteinase activation is an early event in doxorubicin-induced cardiotoxicity.Oncol. Rep. 2004; 11, 505 – 508.
[8]. K.E. van der Vos, P.J. Coffer. The extending network of FOXO transcriptional targetgenes, Antioxid. Redox Signal. 14 (2011) 579–592.
[9]. L.Y. Zhou, R.B. Li, C.Y. Liu, et al. Foxo3a inhibits mitochondrial fission and protectsagainst doxorubicin-induced cardiotoxicitybysuppressing MIEF2.Free RadicBiol Med. 2017;104:360-370.
[10]. D.A. Chistiakov, A.N. Orekhov, Y.V. Bobryshev. The impact of FOXO-1 to cardiac pathology in diabetes mellitus anddiabetes-related metabolic abnormalities. International Journal of Cardiology 245 (2017) 236–244.
[11]. R. Geertman, A. McMahon, E.L.Sabban. Cloning and characterization of cDNAs for novel proteins with glutamic acid-proline dipeptide tandem repeats. Biochim. Biophys. Acta. 1996; 1306: 147-152.
[12]. D. Lu, J. Liu, J Jiao, et al. 2013. Transcription factor Foxo3a prevents apoptosis by regulating calcium through the apoptosis repressor with caspase recruitment domain. J. Biol. Chem. 2013; 288: 8491-8504.
[13]. S. Letasiova, S. Antiproliferative activity of berberine in vitro and in vivo. BiomedPap Med FacUnivPalacky Olomouc Czech Repub, 2005. 149(2): p. 461-3.
[14]. T.L.Serafim. Different concentrations of berberine result in distinct cellularlocalization patterns and cell cycle effects in a melanoma cell line. Cancer ChemotherPharmacol,2008. 61(6): p. 1007-18.
[15]. N. Tong.Berberine sensitizes mutliple human cancer cells to the anticancer effectsof doxorubicin in vitro. OncolLett, 2012. 3(6): p. 1263-1267
[16]. J. Tabeshpour, M. Imenshahidi, and H. Hosseinzadeh. "Areview of the effects of Berberis vulgaris and its major component, berberine, in metabolic syndrome," Iranian Journal ofBasic Medical Sciences, vol. 20, no. 5, pp. 557–568, 2017.
[17]. J. Ming, S. Xu, C. Liu, et al. "Effectivenessand safety of Bifidobacteria and berberine in people withhyperglycemia: study protocol for a randomized controlledtrial," Trials, vol. 19, no. 1, p. 72, 2018.
[18]. I.E. Allijn, B.S. Czarny , X.Y. Wang, et al. Liposome encapsulated berberine treatment attenuates cardiac dysfunction after myocardial infarction J Control Release 2017 Feb 10;247:127-133
[19]. Y.J. Zhang, S.H. Yang, M.H Li, et al.Berberine attenuates adverse left ventricular remodeling and cardiac dysfunction after acute myocardial infarction in rats: role of autophagyClinExpPharmacolPhysiol2014 Dec;41(12):995-1002
[20]. Y.J. Wang, J.Z. Liu, A. Ma, et al. Cardioprotective effect of berberine against myocardial ischemia/reperfusion injury via attenuating mitochondrial dysfunction and apoptosis。Int J ClinExp Med. 2015; 8(8):14513-9
[21]. W.G Chang, M. Zhang, J. Li, etal.Berberine attenuates ischemia-reperfusion injury via regulation of adenosine-5'-monophosphate kinase activity in both non-ischemic and ischemic areas of the rat heart.Cardiovasc Drugs Ther. 2012;26(6):467-78
[22]. G.L.Zhao 1, L.M. Yu, W.Li Gao, et al. Berberine protects rat heart from ischemia/reperfusion injury via activating JAK2/STAT3 signaling and attenuating endoplasmic reticulum stress. ActaPharmacol Sin.2016;;37(3):354-67
[23]. M. Abudureyimu , W.J Yu , etal.Berberine Promotes Cardiac Function by Upregulating PINK1/Parkin-Mediated Mitophagy in Heart Failure. Front Physiol. 2020; 25;11:565751
[24]. Y.Z. Wu, L. Zhang, Z.X. Wu, T.T. Shan, et al.Berberine Ameliorates Doxorubicin-Induced Cardiotoxicity via aSIRT1/p66Shc-Mediated Pathway.Oxid Med Cell Longev. 2019;2019:2150394
[25]. A.R. Coelho, R. Tatiana, R. Martins, et al.Berberine-induced cardioprotection and Sirt3 modulation in doxorubicin-treated H9c2 cardiomyoblasts.BiochimBiophysActaMol Basis Dis2017;1863(11):2904-2923
[26]. C.Y Meng, Y.F. Han, Y.L Liu, et al. Resveratrol alleviate the injury of mice liver induced by cadmium sulfide nanoparticlesKaohsiung J Med Sci. 2019 May;35(5):297-302.
[27]. M.H. Liu, C.Yuan, J. He, et al. Resveratrol protects PC12 cells from high glucose-induced neurotoxicity via PI3K/Akt/FoxO3a pathway. Cell MolNeurobiol. 2015;35(4):513-22
[28]. L.F. Wang, S.W. Su, L. Wang, et al. Tert-butylhydroquinone ameliorates doxorubicin-inducedcardiotoxicity by activating Nrf2 and inducing theexpression of its target genes. Am J Transl Res 2015;7(10):1724-1735.
[29]. R. Zhang, Y.Niu, Y. Li, et al. Acute toxicity study of the interaction between titanium dioxide nanoparticles and lead acetate in mice. Environ. Toxicol. Pharmacol. 2010; 30: 52-60.
[30]. Z.Z. Wang, Y. Liu, X.H. Liu, et al. Activation of forkhead box O3a by mono(2-ethylhexyl)phthalate and its role in protection against mono(2-ethylhexyl)phthalate-induced oxidative stress and apoptosis in human cardiomyocytes. J ApplToxicol. 2020 Oct 8. doi: 10.1002/jat.4070
[31]. A.U. Buzdar, C. Marcus, T.L. Smith, et al. Early and delayed clinical cardiotoxicityof doxorubicin. Cancer. 1985;55:2761-2765
[32]. N. Nozaki, T. Shishido, Y. Takeishi, et al. Modulation of doxorubicin-induced cardiacdysfunction in toll-like receptor-2-knockout mice. Circulation. 2004;110:2869-2874
[33]. R.S.Nho, P.Hergert. FoxO3a and disease progression. World J. Biol. Chem. 2014; 5: 346-354.
[34]. X. Li, N. Du, Q. Zhang, et al. Microrna-30d regulates cardiomyocytepyroptosis by directlytargeting foxo3a in diabetic cardiomyopathy. Cell death & disease. 2014;5:e1479
[35]. Y. Zhou, C. Liang, F. Xue F, etal.Salinomycindecreasesdoxorubicin resistance in hepatocellular carcinoma cells by inhibiting the beta-catenin/tcfcomplex association via foxo3a activation. Oncotarget. 2015;6:10350-10365
[36]. S.F. Sampaio, A.F. Branco, A. Wojtala, et al. P66shcsignaling is involved in stress responses elicited by anthracycline treatment of ratcardiomyoblasts. Archives of toxicology. 2016;90:1669-1684
[37]. A. Sengupta, J.D. Molkentin, J.H.Paik, et al.FoxO transcription factors promote cardiomyocyte survival upon induction of oxidative stress. J. Biol. Chem. 2011, 286: 7468-7478.
[38]. K.Hirofumi, N. Hidetoshi, K. Satoruet, et al. Antioxidants improve the phenotypes of dilated cardiomyopathy and muscle fatigue in mitochondrial superoxide dismutase-deficient mice. Molecules, 2013, 18: 1383-1393

[39]. J Yao，W. Kong W，J. Jiang. Learning from berberine: Treating chronic diseases through multiple targets. Sci China Life Sci， 2015, 58 (9): 854–859.
[40]. W. He，C. Wang，Y Chen，et al., Berberine attenuates cognitive impairment and ameliorates tau hyperphosphorylation by limiting the self -perpetuating pathogenic cycle between NF-κB signaling, oxidative stress and neuroinflammation. Pharmacol Rep，2017，69(6):1341-1348.
[41]. K. Wu, Q. Yang, Y. Mu et al., "Berberine inhibits the proliferation of colon cancer cells by inactivating Wnt/β-cateninsignaling," International Journal of Oncology, vol. 41, no. 1,pp. 292–298, 2012.
[42]. J. B. Patil, J. Kim, and G. K. Jayaprakasha, "Berberineinduces apoptosis in breast cancer cells (MCF-7) throughmitochondrial-dependent pathway," European Journal ofPharmacology, vol. 645, no. 1-3, pp. 70–78, 2010.
[43]. Q. Chen，R Qin，Y.Fang，et al.,Berberine Sensitizes Human Ovarian Cancer Cells to Cisplatin Through miR-93/PTEN/Akt Signaling Pathway.CellPhysiolBiochem，2015，36(3)：956-965.
[44]. A. Mittal, S. Tabasum, and R. P. Singh, "Berberine in combination with doxorubicin suppresses growth of murine melanomaB16F10 cells in culture and xenograft," Phytomedicine, vol. 21, no. 3, pp. 340–347, 2014.
[45]. N. Tong, J. Zhang, Y. Chen et al., "Berberine sensitizes mutliplehuman cancer cells to the anticancer effects of doxorubicinin vitro," Oncology Letters, vol. 3, no. 6, pp. 1263–1267, 2012.