17β-estradiol protects against doxorubicin-induced cardiotoxicity in male Sprague-Dawley rats by regulating NADPH oxidase and apoptosis genes

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Abstract. Doxorubicin (DOX) is one of the most effective chemotherapeutic agents for the treatment of a number of malignancies. However, its use is limited by serious cardiotoxic effects, for which there are currently no reliable pharmacologic therapies. Estrogen has exhibited protective effects against cardiac stressors in male and female animal models; however, its effects on DOX-induced cardiotoxicity remain unknown. High mortality and morbidity rates have been observed in patients with cancer worldwide, and DOX is often administered to a greater number of men than women. Therefore, the present study employed male Sprague-Dawley rats to evaluate the protective effects of 17β-estradiol (E2) against DOX-induced cardiotoxicity. A total of 4 mg/kg DOX was administered to 14-week-old male Sprague-Dawley rats by intraperitoneal injection twice a week for 2 weeks. At 3 weeks following the first injection of DOX, an echocardiographic study revealed that DOX administration significantly decreased cardiac ejection fraction and fractional shortening by 20 and 29%, respectively, when compared with the vehicle-treated control rats ($P<0.05$). This was associated with decreased heart weight, myofibrillar disorganization and myofiber loss. The serum biomarkers for heart injury, including alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase and creatine kinase, were increased in DOX vs. vehicle-treated rats ($P<0.05$). E2 treatment by a daily subcutaneous injection of 2 mg/kg body weight attenuated the cardiotoxic effects of DOX. In addition, E2 treatment inhibited the DOX-induced increase in the expression of cardiac genes, nicotinamide adenine dinucleotide phosphate oxidase (NOX) 2, NOX4, B-cell lymphoma 2-associated X protein and caspase 3. These results demonstrate that E2 treatment may protect the heart against DOX-induced cardiotoxicity in male rats potentially through the regulation of NOX2, NOX4 and apoptosis genes.

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Introduction

The anthracycline anticancer drug doxorubicin (DOX) is one of the most effective and frequently used chemotherapeutic agents in the treatment of a number of human malignancies (1,2). However, its clinical use is limited by side effects, particularly cardiotoxicity, which is a major adverse effect. It has been previously reported that ~11% patients treated with DOX develop acute cardiotoxicity within 2-3 days of administration, and ~1.7% patients develop chronic DOX cardiotoxicity (3,4). The prognosis of patients diagnosed with congestive heart failure is poor (~50% mortality/year), and there are currently no reliable pharmacological therapies available (3,4). Dexrazoxane treatment may limit the cardiotoxic potential of high-dose DOX treatment (>300 mg/m^2), however, this agent also demonstrates side effects, including second primary malignancies (5). Therefore, an improved understanding of the underlying mechanisms associated with DOX-induced cardiotoxicity is required in order to develop a more specific and effective treatment with fewer or no side effects.

Estrogen, primarily produced in the ovaries of premenopausal women, is the primary female sex hormone, which is responsible for the development and regulation of the female reproductive system and secondary sex characteristics. The association between estrogen and the pathogenesis of cardiovascular diseases has been widely investigated (6-9). The incidence of cardiovascular disease is lower in premenopausal women than in men, and significantly increases in postmenopausal women, which indicates that estrogen may have cardioprotective effects (6). In animal studies, ovariectomies inhibited the female-specific protection against volume-induced cardiac remodeling (7), and attenuated the effect of age on ventricular remodeling in rats (8). By contrast, estradiol administration to ovariectomized female rodents attenuated hypertrophy associated with cardiac pressure overload (9) and aging (8).

Although the cardioprotective effects of estrogen have been studied extensively in females, it has not been fully investigated in males. As in postmenopausal women, men produce estrogen in a number of extragonadal sites, including fat tissue, the liver, brain and adrenal glands (10). Estrogen receptors have been identified in female and male hearts (11-13). Previous studies have indicated that estrogen may serve an important role in the maintenance of cardiac structure and function in men and male animals (14,15). Clinical results have demonstrated that an imbalance of circulating estradiol may be associated with increased mortality in men with chronic systolic heart failure (14). In addition, a previous study demonstrated that 17β-estradiol (E2) may promote survival in male mice with cardiomyopathy (15).

The worldwide mortality and morbidity rates for cancer are high, and DOX is often administered to a greater number of males than females (16). Therefore, it is important to investigate the effects of estrogen on DOX-induced cardiotoxicity in males. The aim of the present preclinical study was to evaluate the protective effects of E2 on DOX-induced cardiac injury and the associated mechanisms in male Sprague-Dawley rats.

Materials and methods

Animals. A total of 26 male Sprague-Dawley rats (age, 14 weeks; average body weight, 402±17 g) were obtained from Shandong University School of Medicine Laboratory Animal Center (Jinan, China). All animal study protocols were approved by the Institutional Animal Research and Ethics Committee of Shandong University. The animals were housed at 2 rats/cage in a light-controlled environment at 18-22˚C, with 12 h light/dark cycles, 50±15% humidity, and with access to food and water *ad libitum* throughout the experimental period.

Experimental protocol. Rats were randomly assigned into the following three groups: The control group (n=9), the DOX-treated group (DOX-V group; n=8), and the DOX plus E2-treated group (DOX-E2; n=9). Doxorubicin hydrochloride (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in saline, and 4 mg/2 ml/kg body weight was administered by intraperitoneal injection twice per week for 2 weeks, with a cumulative dose of 16 mg/kg. Age-matched rats injected with saline (2 ml/kg) were used as controls. A total of 2 mg/kg body weight/day E2 (water soluble; Sigma-Aldrich; Merck KGaA) was administered subcutaneously, commencing at 3 days prior to DOX treatment until sacrifice. Rats were then maintained in animal housing until 3 weeks following the first DOX injection.

At the end of the experiment, and following echocardiographic evaluation, rats were euthanized at 3 weeks following the first DOX injection, via exsanguination by cardiac puncture while under ketamine/xylazine anesthesia (ketamine HCL 60 mg/kg; xylazine HCL 5 mg/kg; Sigma-Aldrich; Merck KGaA). The serum was used to examine the different parameters associated with cardiac damage. Whole hearts were isolated and further dissected to isolate the left ventricle. Tissue weights and tibial lengths were measured using an analytical scale and a micrometer, respectively. The left ventricle was divided into sections for RNA, western blot, and histological analyses.

Evaluation of left ventricular systolic function by M-mode echocardiography. Rats were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine mixture at 1 week following the last injection of DOX. The chest was shaved, and the animals were positioned on their left side. Diastolic interventricular septum thickness (IVSTd), systolic interventricular septum thickness (IVSTs), ejection fraction (EF) and fractional shortening (FS) parameters were measured as described previously (17), using a Philips 5500 echocardiography system and a 12 MHz phased array probe (Philips Medical Systems, Inc., Bothell, WA, USA).

Cardiac injury-associated blood biomarkers. Different parameters associated with cardiac injury, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and creatine kinase (CK) were assayed using commercial kits from Sigma-Aldrich (Merck KGaA) as follows: ALT (cat. no. MAK052), AST (cat. no. MAK055), LDH (cat. no. MAK066), and CK activity assay kits (cat no. MAK116). Enzyme activities were measured according to the manufacturer's instructions using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA, USA), and expressed as international units (U/l).

Histological examination of tissues. The left ventricle sections were fixed in 10% formalin at room temperature for 24 h, dehydrated through a graded alcohol series and embedded in paraffin wax. Paraffin sections were cut into 4 μm thick sections, and were deparaffinized by immersion in xylene and rehydrated. Slides were stained with hematoxylin and eosin (H&E) at room temperature (0.5% haematoxylin for 5 min and 0.1% eosin for 1 min), dehydrated using a graded alcohol series, immersed in xylene, and mounted for histological examination using a Zeiss Axiophot microscope (Zeiss AG, Oberkochen, Germany).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR was used to measure mRNA levels in the tissues. Total RNA was extracted from frozen left ventricles using TRIzol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA), and processed according to the manufacturer's recommendations. Complementary first strand DNA was synthesized from 2 μg of oligo(dT)-primed total RNA, using the Omniscript RT kit (Qiagen, Inc., Valencia, CA, USA). Relative quantification of mRNA levels by RT-qPCR was performed using a SYBR green PCR kit (Qiagen, Inc.). Amplification and detection were performed using the ABI 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR amplification conditions were as follows: Denaturation at 95˚C for 15 min followed by 40 cycles of denaturation at 94˚C for 15 sec, annealing at 60˚C for 1 min and extension at 72˚C for 30 sec. The PCR products were quantified using quantification cycle (C_q) values, which were defined as the fractional cycle number at which the fluorescence signal exceeded a fixed threshold. ΔC_q represented the difference in expression between the target gene and the

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer	Primer sequence	Product size (bp)	Accession number
NOX2	Sense	5'-GTGGAGTGGTGTGTGAATGCC-3'	164	NM_023965
	Antisense	5'-ATGCCAGCCAACCGAGTCACA-3'		
NOX4	Sense	5'-CTGCATCTGTCCTGAACCTCAA-3'	101	XM_008759643
	Antisense	5'-TCTCCTGCTAGGGACCTTCTGT-3'		
BAX	Sense	5'-GAGCGGCTGCTTGTCTGGAT-3'	161	NM_017059
	Antisense	5'-CAAGGCAGCAGGAAGCCTCA-3'		
Caspase 3	Sense	5'-GACTGCGGTATTGAGACAGA-3'	209	NM_012922
	Antisense	5'-CGAGTGAGGATGTGCATGAA-3'		
GAPDH	Sense	5'-GGCAAGTTCAATGGCACAGT-3'	151	NM_017008
	Antisense	5'-TGGTGAAGACGCCAGTAGACTC-3'		

NOX, nicotinamide adenine dinucleotide phosphate oxidase; BAX, B-cell lymphoma 2-associated X protein.

GAPDH endogenous control. The normalized relative target mRNA level ($\Delta\Delta C_q$) was calculated using the following equation: $\Delta\Delta C_q = \Delta C_q$ (treated animals)-ΔC_q (control animals). The normalized relative target mRNA level in each sample was calculated as $2^{-\Delta\Delta Cq}$ (18). RT-qPCR was performed in duplicate and a non-template control was included in each run to test for contamination. Sequence-specific oligonucleotide primers were designed according to published GenBank sequences (https://www.ncbi.nlm.nih.gov/genbank; Table I).

Western blot analysis. Left ventricle homogenates were prepared using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Protein samples (20 μg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes for western blot analysis. Immunoblots were probed using antibodies against nicotinamide adenine dinucleotide phosphate oxidase (NOX) 2 (1:170 dilution; cat. no. ab129068; Abcam, Cambridge, UK), NOX4 (1:200 dilution; cat. no. ab109225; Abcam), B-cell lymphoma 2-associated X protein (BAX; 1:200 dilution; cat. no. sc-6236; Santa Cruz Biotechnology, Inc.), and caspase 3 (1:200 dilution; cat. no. sc-70497; Santa Cruz Biotechnology, Inc.). GAPDH (1:1,000 dilution; cat. no. ab8245; Abcam) was used as a loading control. All the antibodies were incubated with membranes overnight at 4˚C. Horseradish peroxidase-conjugated secondary antibodies (1:1,000 dilution; cat. no. ab6721 for rabbit, cat. no. ab6728 for mouse; Abcam) were incubated at room temperature for 1 h and immune complexes were visualized by an enhanced chemiluminescence (ECL) Western Blotting Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The bands were digitized using MCID image analysis software (version 7.0; InterFocus Imaging Ltd., Cambridge, UK). The density of each band was expressed in arbitrary units and normalized to that of GAPDH.

Statistical analysis. Data are presented as the mean ± standard error. For all endpoints, one-way analysis of variance was used to determine the significant differences among groups. The significance of interactions among the groups was determined using Tukey's post-hoc tests. Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Estrogen attenuates DOX-induced systolic dysfunction. As shown in Fig. 1, the echocardiograph results demonstrated that chronic DOX administration significantly decreased cardiac EF by ~20% ($P<0.05$) when compared with the control. The DOX-induced decrease in cardiac EF was attenuated by E2 treatment (Fig. 1). Similarly, cardiac FS significantly decreased in the DOX-treated rats by 29% ($P<0.05$) when compared to vehicle-treated rats. This effect was significantly attenuated prevented by E2 treatment ($P<0.05$; Fig. 1).

Heart weight, structure and histological alterations. Heart weight, corrected by tibial length decreased by 18% in DOX vs. vehicle control-treated rats ($P<0.05$), and E2 treatment significantly restored heart weight ($P<0.05$; Fig. 2A). Similarly, the echocardiograph results demonstrated that IVSTs significantly decreased in the DOX-treated group when compared with control rats (1.64±0.15 vs. 2.03±0.1 mm; $P<0.05$; Fig. 2B). However, the decrease in IVSTd exhibited by DOX-treated rats was not significantly different when compared with the controls (0.99±0.11 vs. 1.15±0.12 mm; $P=0.13$; Fig. 2C). The decrease in IVSTs and IVSTd induced by DOX treatment were inhibited by E2 treatment, however, this did not reach statistical significance (Fig. 2B and C).

Histopathological evaluation by H&E staining of cardiac tissues revealed that DOX induced disorganization of myofibrillar morphology, myofibrillar loss in ~80% cells and cytoplasmic vacuolization (Fig. 2D). In rats treated with DOX and supplemented with E2, histopathological examination with H&E staining revealed similar myocardial fibers and architecture to that observed in the control rats (Fig. 2D).

Serum biomarkers for cardiac injury. Consistent with the echocardiography results and histopathological evaluations,

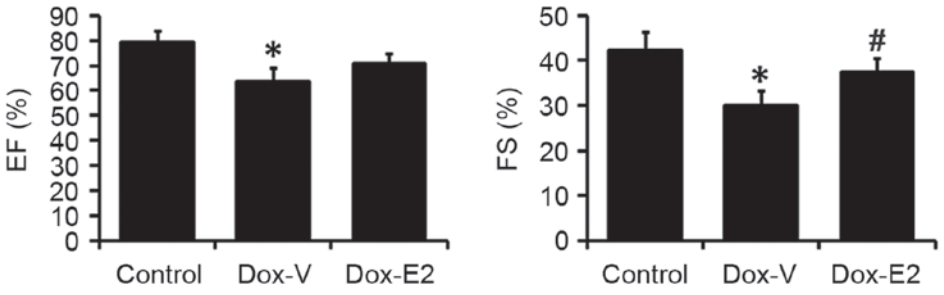

Figure 1. Estrogen attenuated DOX-induced systolic dysfunction. Cardiac EF and FS were significantly lower in DOX-treated male Sprague-Dawley rats when compared with control rats, and E2 treatment attenuated this effect. Values are presented as mean ± standard error (n=8/9). *P<0.05 vs. control; #P<0.05 vs. DOX-V-treated group. DOX, doxorubicin; DOX-V, DOX + vehicle treatment; EF, ejection fraction; FS, fractional shortening; E2, 17β-estradiol.

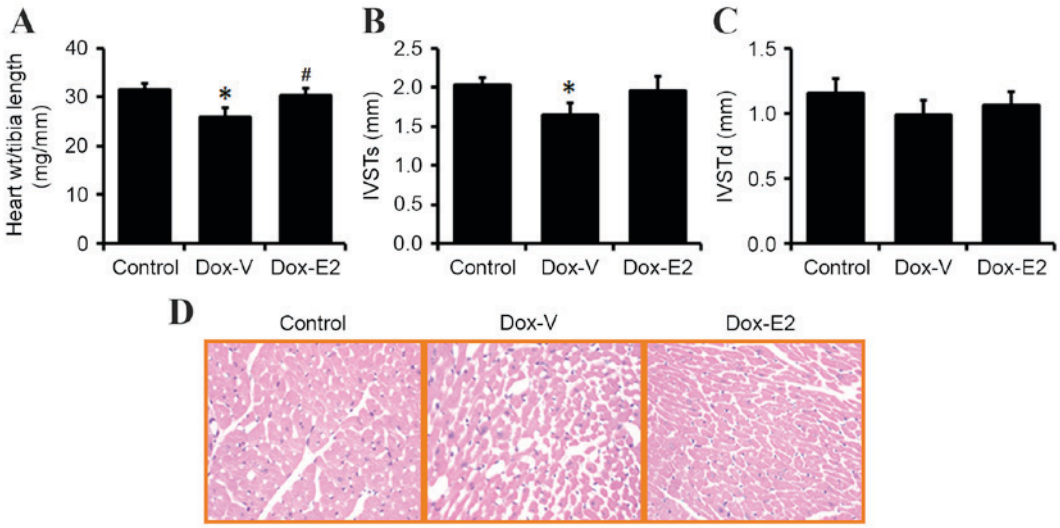

Figure 2. Estrogen attenuated DOX-induced heart injury. (A) The heart weight of control and DOX-treated male Sprague-Dawley rats following vehicle or E2 treatment. Echocardiograph-derived (B) IVSTs and (C) IVSTd measurements are indicated. (D) Representative images of hematoxylin and eosin staining of left ventricles (original magnification, x200). Values are presented as the mean ± standard error (n=8/9). *P<0.05 vs. control; #P<0.05 vs. DOX-V-treated group. DOX, doxorubicin; IVSTs, systolic interventricular septum thickness; IVSTd, diastolic interventricular septum thickness; DOX-V, DOX + vehicle treatment; E2, 17β-estradiol.

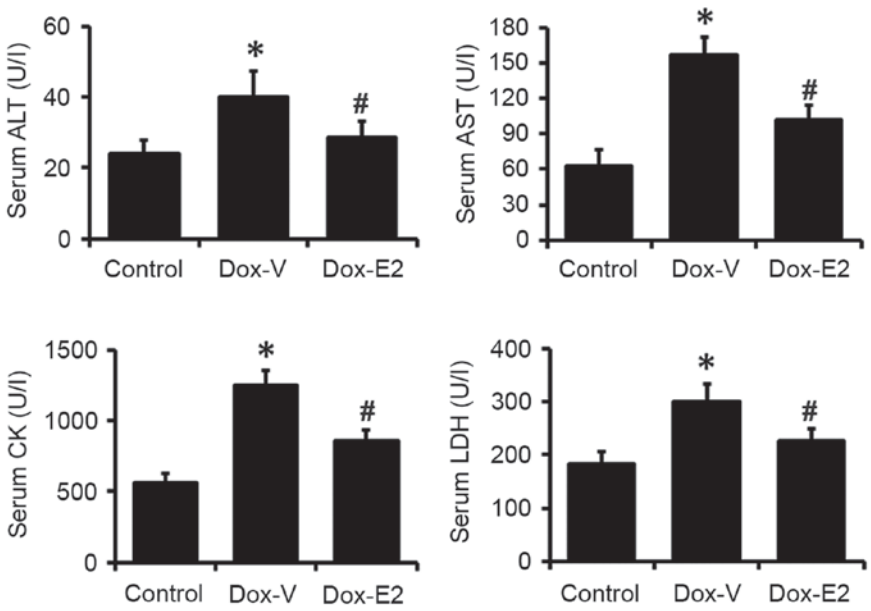

Figure 3. Level of serum biomarkers, ALT, AST, CK and LDH, for cardiac injury. Values are presented as the mean ± standard error (n=8/9). *P<0.05 vs. control; #P<0.05 vs. DOX-V-treated group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase, CK, creatine kinase; DOX, doxorubicin; DOX-V, DOX + vehicle treatment; E2, 17β-estradiol.

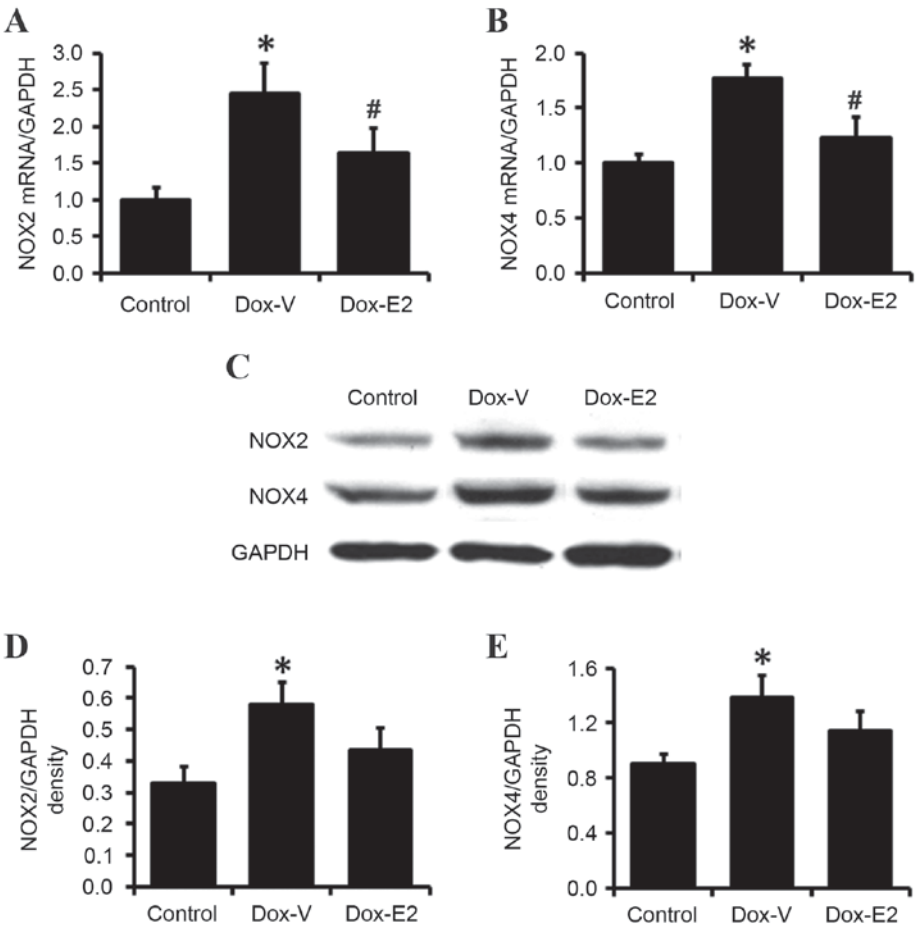

Figure 4. E2 inhibits DOX-induced increases in cardiac NOX2 and NOX4 expression. Cardiac (A) NOX2 and (B) NOX4 mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. (C) Representative western blotting images of NOX2 and NOX4 protein expression. Band densities of (D) NOX2 and (E) NOX4 were quantified and normalized to GAPDH. Values are presented as the mean ± standard error (n=8/9). *P<0.05 vs. control; #P<0.05 vs. DOX-V-treated group. DOX, doxorubicin; NOX, nicotinamide adenine dinucleotide phosphate oxidase; DOX-V, DOX + vehicle treatment; E2, 17β-estradiol.

all serum biomarkers for cardiac injury assayed in the present study, including ALT, AST, LDH and CK, were significantly increased by 63 and 150% in the DOX-treated group when compared with vehicle control-treated rats (P<0.05; Fig. 3). This effect was significantly attenuated by E2 treatment (P<0.05; Fig. 3).

Cardiac NOX2 and NOX4 gene expression. In order to investigate the mechanisms underlying the cardioprotective effects of E2 on DOX-induced cardiotoxicity further, NOX2 and NOX4 gene expression was measured at the mRNA and protein levels by RT-qPCR and western blot analyses, respectively. As shown in Fig. 4A and B, cardiac NOX2 and NOX4 mRNA levels increased by 144 and 77% in DOX-treated rats, respectively, when compared to control rats (P<0.05). E2 treatment significantly attenuated the DOX-associated increase in cardiac NOX2 and NOX4 mRNA levels (P<0.05; Fig. 4A and B). These results were confirmed by western blot analysis, which demonstrated that E2 inhibited DOX-induced increases in cardiac NOX2 and NOX4 protein expression levels (Fig. 4C-E).

Cardiac BAX and caspase 3 gene expression. The expression levels of the apoptosis-associated genes BAX and caspase 3 were measured in the heart tissues of rats from all experimental groups. As expected, RT-qPCR and western blot results demonstrated that DOX significantly increased BAX and caspase 3 mRNA and protein expression levels in heart tissues (P<0.05; Fig. 5), which was significantly inhibited by E2 treatment (P<0.05; Fig. 5).

Discussion

DOX, an effective anthracycline antitumor antibiotic, is used extensively to treat a number of malignancies, including rhabdomyosarcoma, acute leukemia and Hodgkin's disease (1,2). However, due to its serious cardiotoxic effects, DOX has limited clinical use (3,4). Symptomatic congestive heart failure is the most severe complication of DOX-based chemotherapy, with an incidence rate of 5 to 48%, depending on the cumulative dose received (3,4). Therefore, understanding the underlying mechanisms of DOX-induced cardiotoxicity is critical for the development of specific and effective strategies to target this life-threatening side effect of cancer treatment. In addition, investigating the effect of estrogen on DOX-induced cardiac injury in males is of greater importance, as the use of DOX, and cancer mortality and morbidity rates are greater in males than in females (16).

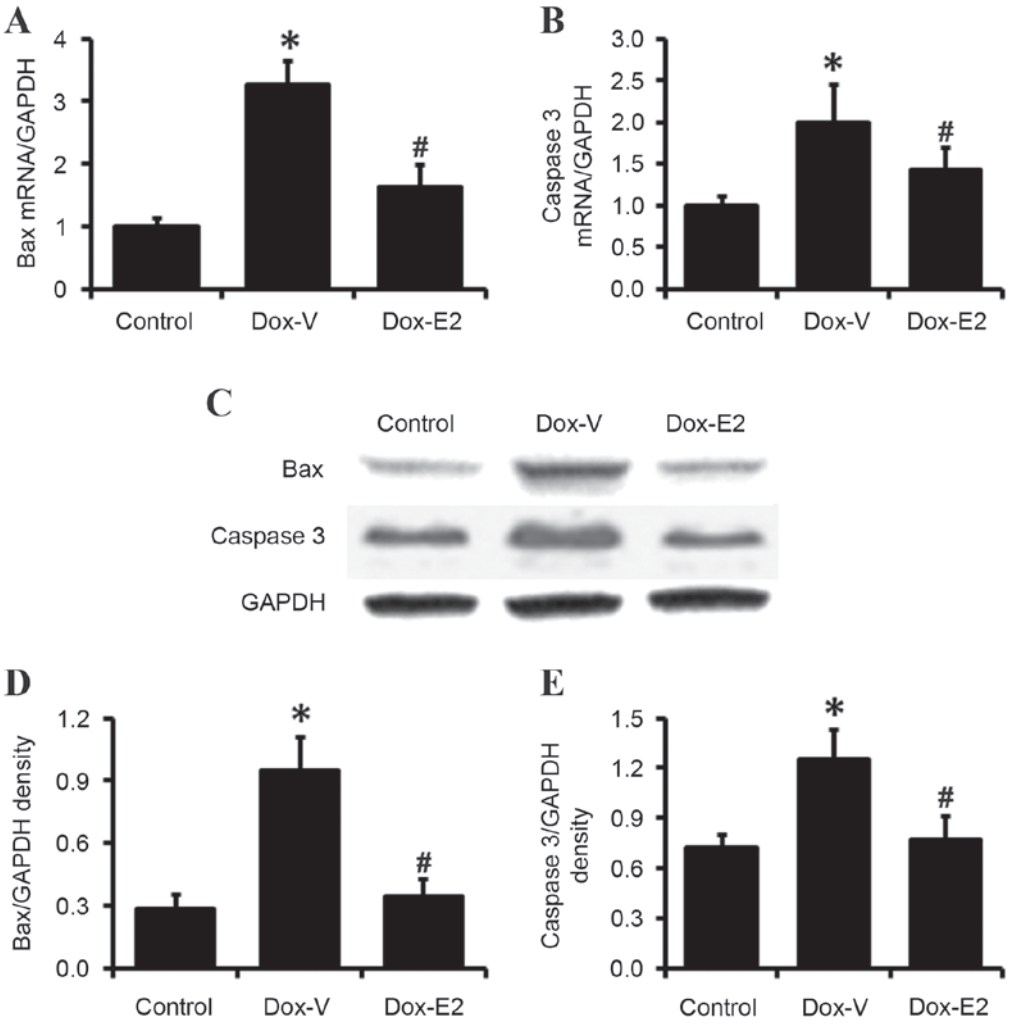

Figure 5. E2 inhibits DOX-induced increases in cardiac BAX and caspase 3 expression. Cardiac (A) BAX and (B) caspase 3 mRNA levels were determined by reverse transcription-quantitative polymerase chain reaction. (C) Representative western blotting images of BAX and caspase 3 protein expression. Band densities of (D) BAX and (E) caspase 3 were quantified and normalized to GAPDH. Values are presented as the mean ± standard error (n=8/9). *P<0.05 vs. control; $^{\#}$P<0.05 vs. DOX-V-treated group. DOX, doxorubicin; BAX, B-cell lymphoma 2-associated X protein; DOX-V, DOX + vehicle treatment; E2, 17β-estradiol.

The results of the present study have provided novel and significant evidence that E2 treatment may protect the heart from DOX-induced cardiotoxicity in male rats. These cardioprotective effects of E2 may be mediated via the regulation of NOX2, NOX4 and apoptosis-associated genes. The results may provide sufficient experimental evidence to support the introduction of monitoring circulating estrogen levels in male patients undergoing treatment with DOX.

The cardioprotective effects of estrogen in females have been investigated in detail in clinical and experimental studies (19,20). Similar to females, estrogen is produced in males, and its receptors have been detected in the hearts of male animals and humans (11-13). However, few studies conducted thus far, have determined the role of estrogen in the male heart. It is possible that the abnormal circulating levels of estrogen in men may contribute to the pathogenic processes and prognoses of a variety of cardiac diseases. A clinical study involving 501 male patients with chronic heart failure demonstrated that low and high concentrations of circulating estradiol were significant predictors of a poor prognosis, independent of gonadal and adrenal androgen deficiencies and conventional clinical prognostic indicators (14). A prospective population-based study with a 4.5-year follow-up period, involving healthy elderly men aged 69-80 years, demonstrated that elderly men with low serum estradiol levels exhibited a significantly increased risk of mortality (21). These results reveal the importance of estrogen levels in healthy men, particularly elderly men, and in male patients with cardiac diseases. In the present study, the dose of E2 used was based on previous reports (22,23). However, more studies are needed to determine the dose-response and the estrogen levels in male animals under physiological and pathological conditions. Chronic E2 treatment at the dose of 2 mg/kg body weight/day attenuated the DOX-induced cardiac systolic dysfunction associated with oxidative stress and alterations to apoptosis-associated gene expression in male rats. The present study indicates that the focus of future research should involve investigating estrogen levels in male patients undergoing DOX treatment. In addition, further studies may be necessary to investigate the potential of estrogen-mediated pathways as a novel target for treating DOX-induced cardiotoxicity.

The major mechanism of DOX-induced cardiotoxicity is associated with the excessive generation of myocardial reactive oxygen species (ROS) and oxidative stress (24-26). Experimental and clinical studies have demonstrated that the adverse cardiac effects of DOX are inhibited by exogenous antioxidant treatment or overexpression of an endogenous antioxidant enzyme (27). In addition, the major source of ROS in the heart is known to be NOX (28,29) and among the 7 isoforms of this enzyme, NOX2 and NOX4 are the primary isoforms expressed in the heart (30). These isoforms have been demonstrated to participate in DOX-induced cardiac ROS generation and heart failure (31,32). It has been reported that oxidative stress modulates a number of key processes underlying DOX-induced cardiotoxicity, including extracellular matrix remodeling, cardiomyocyte apoptosis and altered cardiac contractile properties (33-35). In the present study, E2 treatment inhibited the DOX-induced increase in NOX2 and NOX4 expression in the heart, which may underlie its cardioprotective effects in this male rat model of cardiac injury. This result is consistent with previous findings observed in females, which suggests that estrogen, as an important antioxidant molecule, affects NOX gene expression and activity in animal models (36-38).

NOX-dependent ROS production induces cardiomyocyte apoptosis, which may contribute to DOX-induced cardiotoxicity (31,39,40). As matured myocardial cells are terminally differentiated cells and are unlikely to regenerate when suffering from lethal injury (41), excessive apoptosis induces a decrease in myocardial cells and leads to cardiac dysfunction, which eventually progresses to heart failure. In the present study, DOX decreased heart weight and echocardiography-derived IVSTs, of which the mechanism might be due to the DOX-induced cardiomyocyte apoptosis. While apoptosis was not directly determined in this study, expression levels of apoptosis-related proteins BAX and caspase 3 were increased following DOX treatment. Treatment with E2 attenuated these DOX-induced alterations in male rats, suggesting that E2 may protect the heart from DOX-induced cardiotoxicity potentially via the inhibition of the activated cardiac NOX/ROS/apoptosis pathway induced by DOX treatment.

Estrogen is produced and binds to associated receptors in cardiac tissues in males as well as females. However, the role of estrogen in healthy men, and in the pathogenesis and prognosis of cardiac diseases may be severely underestimated. The present study demonstrated that E2 treatment inhibited DOX-induced cardiotoxicity in male rats, and was associated with decreased cardiac NOX2, NOX4 and apoptosis gene expression. The results may provide a greater understanding of the underlying mechanisms of DOX-induced cardiotoxicity in males and provide experimental evidence for novel therapeutic approaches involving estrogen-mediated pathways in the male heart.

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