Early Administration of Carvedilol Protected Against Doxorubicin-Induced Cardiomyopathy


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Abstract

This study tested for the benefits of early administration of carvedilol as protection against doxorubicin-induced cardiomyopathy. Thirty male-adult B6 mice were categorized into group 1 (untreated control), group 2 [doxorubicin (15 mg/every-other-day for 2-weeks, I.P.), and group 3 [carvedilol (15 mg/kg/day, from day-7 after doxorubicin for 28 days)] and euthanized by day 35 after doxorubicin treatment. By day 35, the left-ventricular injection fraction (LEVF) was significantly lower in group 2 than in groups 1 and 3, and significantly lower in group 3 than in group 1, whereas the LV end-diastolic and LV end-systolic dimensions showed an opposite pattern to LVEF among the three groups. The protein expressions of fibrotic (Smad3, TGF-β), apoptotic (BAX, cleaved caspase 3, PARP), DNA-damage (γ-H2AX), oxidative-stress (oxidized protein), mitochondrial-damage (cytosolic cytochrome-C), heart failure (BNP), hypertrophic (β-MHC) biomarkers of LV myocardium showed an opposite pattern to LVEF among the three groups. The protein expressions of anti-fibrotic (BMP-2, Smad1/5), α-MHC, and phosphorylated-Akt showed an identical pattern to LVEF among the three groups. The microscopic findings of fibrotic and collagen-deposition areas, numbers of γ-H2AX+ and 53BP1+ cells in LV myocardium, exhibited an opposite pattern, whereas the numbers of endothelial cell (CD31+, vWF+) markers showed an identical pattern to LVEF among the three groups. Cardiac stem cell markers (C-kit+, Sca-1+ cells) were significantly progressively increased from group 1 to group 3. Additionally, The in vitro study showed carvedilol treatment significantly inhibited DOX-induced cardiomyoblast DNA (CD90/XRCC1+, CD90/53BP1+ and r-H2AX+ cells) damage. Early carvedilol therapy protected against doxorubicin-induced DNA-damage and cardiomyopathy.
Introduction

Hypertrophic cardiomyopathy can be caused by any disease that increases cardiac afterload and volume overload, some myocardiotropic drugs, and certain primary genetic disorders of the myocardium (Senni et al., 1998; Roura and Bayes-Genis, 2009; Masuda et al., 2012; Maron et al., 2014; Modesto and Sengupta, 2014; Patel et al., 2015). Without appropriate treatment, hypertrophic cardiomyopathy commonly develops into dilated cardiomyopathy and, ultimately, decompensated heart failure (Senni et al., 1998; Roura and Bayes-Genis, 2009; Masuda et al., 2012; Patel et al., 2015).

Doxorubicin (DOX) is used to treat a variety of human neoplasms but is limited by its cardiotoxicity (Blum and Carter, 1974; Von Hoff et al., 1979). Long-term treatment with DOX can cause cardiomyopathy and congestive heart failure (CHF) in a process that involves multiple factors, including the generation of free radicals that further damage cellular membranes (Rajagopalan et al., 1988; Keizer et al., 1990), disturbance of adrenergic function, alterations in intracellular Ca2+ homeostasis (Kim et al., 1989), myocardial cell apoptosis/death (Arola et al., 2000; Wu et al., 2000), and selective inhibition of the expression of cardiac muscle-specific proteins (Jeyaseelan et al., 1997). DOX induced cardiac myocyte apoptosis/death through upregulated caspase 3 and downregulated kinase activities of PI 3-kinase and Akt (Negoro et al., 2001).

Carvedilol, a cardioselective beta blocker/alpha-1 blocker, is widely used to treat hypertension and CHF (Yue et al., 1994; Packer et al., 2002). It reduces morbidity and mortality in CHF (Packer et al., 2002), has antioxidant effects, inhibits lipid peroxidation and reduces mitochondrial toxicity (Tadolini and Franconi, 1998; Arozal et al., 2010; Arozal et al., 2011; Pereira et al., 2011). Therefore, we used an animal model to test the hypothesis that carvedilol would protect against myocardial damage caused by DOX and improved the heart function, through inhibiting DNA damage, fibrosis and apoptosis of the left ventricular (LV) myocardium in mice.
Methods

Ethics

Eight-week old male C57BL/6 mice were purchased from Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan, and housed in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC)-approved animal facility in our hospital with controlled temperature (24 °C), humidity (50% ~ 70%), and light cycle (12/12) for 2 weeks before administration of DOX. Additionally, animals were given ad libitum access to food (FWUSOW INDUSTRY CO., LTD) and autoclaved filtered RO water (ELGA MEDICA Pro Water System, UK). All experimental procedures were approved by the Institute of Animal Care and Use Committee of Kaohsiung Chang Gung Memorial Hospital (IACUC No. 20122101) and performed in accordance with the Guide for the Care and Use of Laboratory Animals [The Eighth Edition of the Guide for the Care and Use of Laboratory Animals (NRC 2011)].

To avoid the possibility that a gender-specific effect differences might increase experimental variation and confound statistical analyses, the current investigations were restricted to male mice only.

Pilot Study of DOX Induced Mouse Cardiomyopathy

Dose-dependency of DOX-induced myocardial damage (i.e., cardiomyopathy) was determined in mice. Male adult C57BL/6 (B6) mice (n=24), weighing 25-30 mg (Charles River Technology, BioLASCO Taiwan Co., Ltd., Taiwan), were grouped to receive stepwise increased DOX doses [0 (group A), 5 (group B), 10 (group C) and 15 (group D) mg/every-other-day for 2-weeks, I.P., total 7 doses]. The regimen and total accumulated dosage of DOX to be utilized in the present study was based on the previous report (Olson et al., 2003; Miyagawa et al., 2010) with some modification. Additionally, to minimize animal handling and possible discomfort associated with IP injections, DOX was administered
every-other-day for 2 weeks.

The mice were anesthetized with an inhaled anesthesia mixture of isoflurane and oxygen 0.8-1 L/min and placed on a temperature-regulated table (25°C) to maintain body temperature. Isoflurane (2.0%) was vaporized at a concentration of 32-36% in a N2/O2 mixture for transthoracic echocardiographic examination. Monitoring of the depth of anesthesia included testing of rear foot reflexes before skin incision. Respiratory pattern, mucous membrane color, and responsiveness to manipulations, and rear foot reflexes were closely surveyed throughout the procedure. After echocardiographic examination or doxorubicin administration, the mice were returned to the animal center before euthanasia.

The mice were euthanized with an overdose of isoflurane (>5.0%, i.e., continue isoflurane exposure until one minute after the animal stopped breathing) by day 28 after DOX administration and each heart harvested for individual study.

The ratio of total heart weight to body weight was significantly lower in group D than in other groups, significantly lower in groups A and B than in group C, but not different between groups A and B (see Results). Additionally, the left ventricular injection fraction (LVEF) showed a similar pattern of ratio of heart weight to body weight among the four groups (60%, vs. 54%, vs. 50%, vs. 47.3%, p<0.001) (see Results). Thus, DOX-induced cardiomyopathy was recreated in the pilot study.

Animal Grouping and Induction of Cardiomyopathy by DOX (15 mg/ Every-other-Day for 2-Weeks) and Rationale for the Carvedilol Dose in the Current Study

To elucidate the impact of carvedilol therapy on preserving LVEF, additional four animals were utilized and categorized into receiving higher-dose (15 mg/kg/day) and lower-dose (5 mg/kg/day) of carvedilol, respectively. The results of the pilot study showed that LVEF was better preserved in animals (n=2) that received 15 mg/kg/day of carvedilol than in animals (n=2) with 5 mg/kg/day of carvedilol orally (i.e., by lavage) (53.0% vs. 49.0%; carvedilol was commenced from day 7 after DOX administration and administered for 4 weeks).
Additionally, previous study has shown that the dosage of carvedilol up to 30 mg/kg daily for rodent was still safe without any side effect (Matsui et al. 1999). Thus, 15 mg/kg/day of carvedilol was used in the present study.

In the present study, prior to DOX administration, the mice (n=30) were equally randomized into group 1 (untreated control, n=10), group 2 (DOX only, I.P.; n=10) and group 3 [DOX + carvedilol (15 mg/kg/day by lavage) commenced from day 7 after DOX administration for 28 days, n=10]. The present study did not provide carvedilol-treated control group was based on the spirits of Guide for the Care and Use of Laboratory Animals (i.e., replace, reduce, and reduce) and the previous study demonstrated that dosage of carvedilol up to 30 mg/kg daily for rodent was safe (Matsui et al. 1999).

In vitro Study for Identifying the Impact of Carvedilol on Protecting the Cardioblasts from DOX-Induced Toxicity

For the in vitro study, H9C2 cells (cardiomyoblast) were purchased from American Type Culture collection (ATCC; No. CRL1446). The cardiomyoblast characteristics of H9C2 cells were verified with the immunofluorescent staining to detect the specific expressions of cardiac troponin T and cardiac sarcomeric α-actinin. Additionally, H9C2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml of penicillin and 100 μg/ml of streptomycin in a T-75 culture flask at 37°C with 5% CO2. For passage, cells at 70% confluence were enzymatically dissociated with 0.25 % trypsin/EDTA and sub-cultured to new flasks with fresh medium.

To determine the impact of carvedilol against the DOX-induced DNA damage, H9C2 cells were first cultured in DMEM culture medium, followed by (1) co-cultured with stepwisely increased DOX (0, 20, 100, 500 nM) for 24 h, (2) co-cultured with stepwisely increased carvedilol (0, 2, 5, 10 μM) for 24 h, or co-cultured with DOX (100 nM) and carvedilol (10 μM) for 24 h and 48 h, respectively. The cells were then collected for individual study.

Functional Assessment by Echocardiography
The procedure and protocol for transthoracic echocardiography was based on our previous report (Chua et al., 2014). Transthoracic echocardiography (Vevo 2100, Visualsonics) was performed in each group prior to and on day 35 after DOX treatment by an animal cardiologist blind to the experimental design. M-mode standard two-dimensional (2D) left parasternal-long axis echocardiographic examination was conducted. Left ventricular internal dimensions [i.e., left ventricular end-systolic diameter (LVESd) and left ventricular end-diastolic diameter (LVEDd)] were measured at mitral valve and papillary levels of the left ventricle, according to the American Society of Echocardiography leading-edge method using at least three consecutive cardiac cycles. Left ventricular ejection fraction (LVEF) was calculated as follows: LVEF (%) = \[ \frac{(LVEDd^3-LVESd^3)}{LVEDd^3} \] x 100%.

Specimen Collection

Mice in each group were euthanized by day 35 after DOX treatment, and heart in each mouse was rapidly removed and then immersed in cold saline. The LV tissues were isolated and divided into three parts for (1) cryosections [embedded in compound (Tissue-Tek, Sakura, Netherlands], (2) paraffin sections (fixed with 10% formalin), and (3) protein examination (stored in -80 °C refrigerator before using), respectively.

Western Blot Study

The procedure and protocol for Western blot analysis were based on our previous reports (Chen et al., 2014a; Chen et al., 2014b). In brief, equal amounts (50 μg) of protein extracts were loaded and separated by SDS-PAGE using acrylamide gradients. After electrophoresis, the separated proteins were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). Nonspecific sites were blocked by incubation of the membrane in blocking buffer [5% nonfat dry milk in T-TBS (TBS containing 0.05% Tween 20)] overnight. The membranes were incubated for 1 hour at room temperature with the indicated primary antibodies [Bax (1: 1000, Abcam), cleaved poly (ADP-ribose) polymerase (PARP) (1:1000, Cell Signaling), caspase 3 (1: 1000, Cell Signaling), Smad3...
(1:1000, Cell Signaling), transforming growth factor (TGF)-β (1: 500, Abcam), Smad1/5 (1:1000, Cell Signaling), bone morphogenetic protein (BMP)-2 (1: 1000, Abcam), phosphorylation of histone H2AX (γ-H2AX) (1:1000, Cell Signaling), alpha myosin heavy chain (α-MHC) (1:300, Santa Cruz), β-MHC (1:1000, Santa Cruz), brain natriuretic peptide (BNP) (1: 800, Abcam), Akt (1:1000, Cell Signaling), Ku-70 (1:1000, Cell Signaling) and cytosolic (1:2000, BD) and mitochondrial (1:2000, BD) cytochrome C]. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin IgG (1:2000, Cell Signaling) was used as a secondary antibody for one hour incubation at room temperature. The washing procedure was repeated eight times within one hour. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and exposed to Biomax L film (Kodak). For the purpose of quantification, ECL signals were digitized using Labwork software (UVP).

In our western blot study for the assessment of specific protein expressions, we loaded the lysate in the same SDS/polyacrylamide gel and then transferred to the PVDF membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane to assess the expressions of the proteins. That means the β-tubulin expression of each lane was used for comparing with the expressions of different proteins on the same lane.

**Oxidative Stress Measurement of LV Myocardium**

The procedure and protocol for assessing the protein expression of oxidative stress have been detailed in our previous reports (Chen et al., 2014a; Chen et al., 2014b). The Oxyblot Oxidized Protein Detection Kit was purchased from Chemicon (S7150). DNPH derivatization was carried out on 6 μg of protein for 15 minutes according to the manufacturer’s instructions. One-dimensional electrophoresis was carried out on 10% SDS/polyacrylamide gel after DNPH derivatization. Proteins were transferred to nitrocellulose membranes which were then incubated in the primary antibody solution
(anti-DNP 1: 150) for 2 hours, followed by incubation in secondary antibody solution (1:300) for 1 hour at room temperature. The washing procedure was repeated eight times within 40 minutes. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) which was then exposed to Biomax L film (Kodak). For quantification, ECL signals were digitized using Labwork software (UVP). For oxyblot protein analysis, a standard control was loaded on each gel.

The IF and IHC Staining

IF staining was performed using respective primary antibodies for the examinations of CD31+ (1:100, Abcam), γ-H2AX+ (1:500, Abcam), Ku-70 (1:100, Abcam), CD90+ (1:100, abcam), XRCC1+ (1:200, Abcam) and 53BP1+ (1:100, Abcam; 1:300, Novus) cells and Actinin-phalloidin (1:500, LuBio Science; 1:500, Life Technologies) in LV myocardium based on our recent study (Rajagopalan et al., 1988; Kim et al., 1989; Arola et al., 2000; Wu et al., 2000). Moreover, IHC staining was performed for examinations of Sca-1+ (1:300, BioLegend) and C-kit+ (1:300, Santa Cruz) cells using respective primary antibodies as described (Sung et al., 2009; Leu et al., 2011; Chen et al., 2014a; Chen et al., 2014b; Chua et al., 2014). Irrelevant antibodies were used as controls in the current study.

Histological Quantification of Myocardial Fibrosis and Collagen Deposition

The procedure and protocol were described in our previous report (Leu et al., 2011; Chua et al., 2014). Masson's trichrome staining was used to identify fibrosis of the LV myocardium. Three serial sections of LV myocardium in each animal at the same levels were prepared at 4 µm thickness by Cryostat (Leica CM3050S). The integrated area (µm²) of fibrosis on each section were calculated using Image Tool 3 (IT3) image analysis software (University of Texas, Health Science Center, San Antonio, UTHSCSA; Image Tool for Windows, Version 3.0, USA). Three randomly selected high-power fields (HPFs) (100 x) were analyzed in each section. After determining the number of pixels in each infarct and fibrotic area per HPF, the numbers of pixels obtained from three HPFs were summated. The procedure was
repeated in two other sections for each animal. The mean pixel number per HPF for each animal was then determined by summating all pixel numbers and dividing by 9. The mean integrated area (µm$^2$) of fibrosis in LV myocardium per HPF was obtained using a conversion factor of 19.24 (1 µm$^2$ represented 19.24 pixels).

To analyze the extent of collagen synthesis and deposition, cardiac paraffin sections (6 µm) were stained with picrosirius red (1% Sirius red in saturated picric acid solution) for one hour at room temperature using standard methods. The sections were then washed twice with 0.5% acetic acid. The water was physically removed from the slides by vigorous shaking. After dehydration in 100% ethanol thrice, the sections were cleaned with xylene and mounted in a resinous medium. High power fields (×100) of each section were used to identify Sirius red-positive area on each section. Analyses of collagen deposition area in LV myocardium were identical to the description for the calculations of the infarct and fibrotic areas.

**Statistical Analysis**

Quantitative data are expressed as means ± SD. Statistical analysis was performed by ANOVA followed by Bonferroni multiple-comparison post hoc test. SAS statistical software for Windows version 8.2 (SAS institute, Cary, NC) was utilized. A probability value <0.05 was considered statistically significant.

**Results**

**In Vitro Studies Identifying Protective Effect of Carvedilol on DNA Damage Induced by DOX (Figures 1, 2, 3 and 4)**

To elucidate the impact of DOX therapy on DNA damage, H9C2 (cardioblast cell line) were co-cultured with stepwise-increased DOX (0, 20, 100, and 500 nM) for 24 h, followed by collection of cells for Western blot. The results showed that the protein expression of γ-H2AX, a marker of DNA-damage, progressively increased, whereas that of Ku-70 and phosphorylated (p)-Akt, two indicators of DNA repair, progressively decreased (Fig. 1).
To assess whether carvedilol treatment would activate DNA repair in H9C2 cells, the cells were co-cultured with stepwise-increased carvedilol concentration (0, 2, 5, and 10 μM) for 24 h. Protein expressions of Ku-70 and p-Akt progressively enhanced as the dose of carvedilol increased (Fig. 1).

To assess whether carvedilol could prevent DNA damage induced by DOX, H9C2 cells were co-cultured with DOX (100 nM) and carvedilol (10 μM) for 24 h and 48 h, respectively. Protein expressions of p-Akt, Ku-70 and TNNI3K, three indicators of DNA repair, were preserved, whereas protein expression of γ-H2AX was suppressed by carvedilol (Fig. 2). Additionally, IF microscopic findings demonstrated that the numbers of CD90/XRCC1+, CD90/53BP1+ (Fig. 3) and r-H2AX+ cells (Fig. 4), three markers of DNA damage, were significantly higher, whereas the number of Ku70+ cells (Fig. 4), an indicator of DNA repair, was significantly lower in DOX-treated group as compared with control group and the group treated with DOX + carvedilol.

**Doxorubicin Treatment of Dose-Dependent Myocardial Damage and Alternation of Heart Weight and LV Function in Living Animals (Figure 5)**

By day 35 after DOX treatment, the protein expressions of Bax, cleaved caspase 3 and cleaved PARP, three indicators of apoptosis in LV myocardium, progressively increased as DOX dose increased (Fig. 5-A to 5-C). Additionally, the lower dose of DOX (i.e., 10 mg) induced notably cardiac hypertrophy, whereas the higher dose of DOX caused significantly lower ratio of heart weight to tibial length (Fig. 5-D) and total heart weight (Fig. 5-E), an indirect indicator of loss of myocardium and cell death. Furthermore, the LVEF was notably progressively decreased as the DOX dose was stepwise increased (Fig. 5-F).

The results of IHC staining showed that the fibrotic area (Fig. 5-G) and collagen-deposition area in LV myocardium (Fig. 5-H) progressively increased as the DOX-dose increased (please see the illustrations of microscopic findings in supplement data). The results of IF staining displayed that 53BP1+ cells (Fig. 5-I) and γ-H2AX+ cells (Fig. 5-J),
two markers of DNA damage, were identical to the expression of fibrosis in LV myocardium (please see the illustrations of microscopic findings in supplement data).

**Carvedilol Therapy Preserved LV Function and Inhibited LV Remodeling by Day 35 after DOX (15 mg/ Every-other-Day for 2-Weeks) Treatment (Figure 6)**

By day 35 after DOX treatment, the LVEF was significantly lower in group 2 (DOX) than in group 1 (untreated control) and group 3 (DOX + carvedilol), and significantly lower in group 3 than in group 1. Conversely, the LVEDd and LVESd showed a reversed pattern of LVEF among the three groups. These findings suggest that carvedilol treatment protected against DOX-induced myocardial damage.

**Carvedilol Prevented Apoptosis, DNA and Mitochondrial Damage Caused by DOX (15 mg/ Every-other-Day for 2-Weeks) Treatment in LV Myocardium (Figure 7)**

By day 35 after 15 mg of DOX therapy, the protein expressions of Bax, cleaved caspase 3 and PARP, γ-H2AX, and cytosolic cytochrome C (i.e., an indicator of mitochondrial damage, were significantly higher in group 2 than in group 1 and group 3, and significantly higher in group 3 than in group 1. On the other hand, mitochondrial cytochrome C, an indicator of mitochondrial integrity, revealed an opposite pattern to apoptosis among the three groups.

**Carvedilol Prevented Fibrosis and Myocardial Hypertrophy caused by DOX (15 mg/ Every-other-Day for 2-Weeks) Treatment in LV Myocardium (Figure 8)**

By day 35 after doxorubicin treatment, the protein expressions of Smad3 and TGF-β were significantly higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. Conversely, the protein expressions of Smad1/5 and BMP-2, two anti-fibrotic biomarkers, exhibited an opposite pattern to fibrosis among the three groups. Additionally, the protein expressions of BNP and β-MHC, two indicators of pressure overload/heart failure, showed an identical pattern of fibrosis among the three groups. Conversely, protein expression of α-MHC, a reverse myocardial hypertrophic biomarker, showed an opposite pattern to β-MHC among the three groups.
Carvedilol Prevented Microscopic Findings of Fibrosis and Collagen Deposition caused by DOX (15 mg/ Every-other-Day for 2-Weeks) in LV Myocardium (Figure 9)

By day 35 after doxorubicin treatment, Mason’s trichrome staining showed that the fibrotic area was significantly higher in group 2 than in groups 1 and 3, and significantly higher in group 3 than in group 1. Additionally, the results of Sirius red staining exhibited that the collagen deposition area displayed an identical pattern of fibrosis among the three groups.

Cardvedilol Prevented the Expressions of $\gamma$-H2AX+ and 53BP1+ cells Caused by DOX (15 mg/ Every-other-Day for 2-Weeks) Expressions and Upregualtion of Ku-70+ cell Expression in LV Myocardium (Figure 10)

By day 35 after DOX treatment, the numbers of $\gamma$-H2AX+ and 53BP1+ cells, two indices of DNA damage markers, were significantly increased in group 2 compared to groups 1 and 3, and significantly higher in group 3 than in group 1. On the other hand, the cellular expression of Ku-70, a DNA repair biomarker, exhibited an opposite pattern of DNA damage markers among the three groups.

Carvedilol Therapy Enhanced the Expressions of Cardiac Stem Cells and Endothelial cells in LV Myocardium (Figure 11)

By day 35 after DOX (15 mg/ every-other-day for 2-weeks) treatment, the numbers of Sca-1+ and C-kit+ cells, two indicators of cardiac stem cells, were significantly higher in group 2 and significantly increased in group 3 compared to group 1. We suggest that increased numbers of cardiac stem cells after DOX treatment could be an intrinsic response to cardiotoxicity and DNA damage. Additionally, carvedilol therapy had an extrinsic capacity to enhance the increase of cardiac stem cells for myocardial repair. Conversely, the number of CD31+ cells, an indicator of endothelial cells, was significantly lower in group 2 than in groups 1 and 3, and significantly lower in group 3 than in group 1.

Discussion
This study, which investigated the protective effect of carvedilol therapy on myocardium against DOX damage, yielded several striking implications. First, DOX caused significant LV dysfunction and increased LV remodeling. Second, DOX-induced LV dysfunction and remodeling were shown to be mainly through fibrosis, apoptosis, DNA damage, mitochondrial dysfunction, and oxidative stress. Third, DOX therapy was associated with cumulative and dose-dependent cardiomyopathy. Fourth, these molecular-cellular and functional perturbations caused by DOX were significantly reversed by carvedilol, suggesting that such therapy could be effective for patients with neoplasms and DOX-induced cardiotoxicity/cardiomyopathy.

Undoubtedly, accumulating doses of DOX therapy is associated with irreversible dilated cardiomyopathy. Once established, medical therapy is mostly ineffective. Therefore, prevention of cardiotoxicity has great clinical importance.

One important finding in the present study was that cardiotoxicity was strongly associated with stepwise increases in DOX dosage. Additionally, cardiotoxicity/cardiomyopathy occurred at an early stage of DOX therapy (by day 28). Intriguingly, one clinical observational study previously revealed that subclinical systolic dysfunction occurs in almost 50% of patients early after anthracycline (DOX is one kind of anthracyclines) therapy (Lotrionte et al., 2007). Similarly, another observational study showed that changes in LV longitudinal peak systolic strain and LV remodeling were observed early in patients receiving anthracycline chemotherapy (Poterucha et al., 2012). Accordingly, the results of our experimental study (i.e., preclinical study) support the findings of those previous studies (Lotrionte et al., 2007; Poterucha et al., 2012) (i.e., clinical observational study) that cardiotoxicity quite often developed at an early stage of DOX therapy.

The benefit of carvedilol on preserving heart function has been extensively discussed by numerous clinical observational studies (Kalay et al., 2006; Elitok et al., 2014). The most important finding in the present study was that early administration of carvedilol significantly
preserved LV function and inhibited LV remodeling. Our findings strengthen previous work (Kalay et al., 2006; Elitok et al., 2014) and highlight that early administration is essential for protecting against DOX-induced cardiomyopathy. This finding has important preclinical relevance that can be extrapolated to the clinical setting: early provision of carvedilol for chemotherapy patients may be crucial to prevent cardiotoxicity, which in turn, preserves heart function and inhibit LV remodeling.

The cardiotoxicity/cardiomyopathy caused by DOX is due to multiple mechanisms, including generation of free oxygen radicals (Olson and Mushlin, 1990), apoptosis/cell death (Kalyanaraman et al., 2002), mitochondrial dysfunction (Olson and Mushlin, 1990; Wallace et al., 1997), activation of matrix metalloproteinase (Bai et al., 2004), DNA damage/abnormal protein processing (Shi et al., 2011), and decreased vasculogenesis (Shi et al., 2011). Another important finding in the present study was that the fibrotic area and collagen deposition area were remarkably higher in doxorubicin treated group than in untreated control group. Additionally, both in vitro and in vivo studies exhibited that the DNA damage markers, apoptotic and fibrotic biomarkers, as well as the oxidative stress and mitochondrial dysfunction markers (cytosolic cytochrome C increased and mitochondrial cytochrome C decreased) were substantially increased in doxorubicin treated animals compared with those of untreated control animals. Accordingly, our findings corroborate with the findings of those previous studies (Olson and Mushlin, 1990; Kalyanaraman et al., 2002; Shi et al., 2011). However, all of these perturbation parameters were markedly reversed and those of anti-fibrotic biomarkers were notably increased after carvedilol therapy. Therefore, our findings, in addition to supporting the findings of previous studies (Olson and Mushlin, 1990; Kalyanaraman et al., 2002; Shi et al., 2011), could lend partial explanation to why LV function was preserved and LV remodeling was inhibited in DOX-treated animals after receiving carvedilol therapy. In this way, the results of our studies encourage the early use of carvedilol of patients with the requirement of DOX treatment.
Interestingly, in the present study we found that DOX-induced cardiotoxicity would enhance the generation of cardiac stem cells in myocardium. We suggested that such progenitor cell renewal could be an intrinsic response to myocardial damage for regeneration of myocardium. Of importance was that carvedilol therapy further enhanced this phenomenon of progenitor cell renewal. This finding could, at least in part, explain why carvedilol therapy preserved heart function and abrogated LV remodeling in doxorubicin treated animals.

An essential finding in the present study was that the number of CD31 cells was significantly reduced in doxorubicin treated animals than in those of untreated control group, implicating that DOX therapy not only induced cardiotoxicity but also destroyed endothelial cells/endothelial function and angiogenesis. Importantly, this endothelial cell was significantly reversed (i.e., from 0.5% to 2.5% with an increment of 5.0 times) after carvedilol treatment. This increment in CD31+ cells may implicate that the microcirculation and blood supply in myocardium was preserved after carvedilol treatment, therefore, protected cardiomyocytes against ischemia and death. In this way, our findings could also once again explain why carvedilol treatment preserved LV function and ameliorated LV remodeling.

A principal finding in the present study was that the protein expressions of BNP and β-MHC were significantly enhanced, whereas the protein expression of α-MHC was notably reduced in the doxorubicin group than in untreated control controls. In fact, numerous studies have shown correlation between an increase in circulating levels of BNP and CHF/pressure overload, and prognostic outcome in patients after ischemic myocardial infarction (Wu et al., 2006). Additionally, cardiac hypertrophy is characterized by a switch from α- to β-MHC mRNA expression (i.e. reactivation of fetal gene program) (Sun et al., 2014). Intriguingly, these biomarkers were remarkably reversed after carvedilol therapy. In this way, our findings, in addition to being consistent with that of previous studies (Wu et
al., 2006; Sun et al., 2014), once more explains why carvedilol therapy preserve LV function and ameliorate LV remodeling.

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Authorship Contributions

Participated in research design: Yung-Lung Chen, Sheng-Ying Chung and Hon-Kan Yip

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Contributed new reagents or analytic tools: Han-Tan Chai, Chih-Hung Chen, Chu-Feng Liu and Sarah Chua

Wrote or contributed to the writing of the manuscript: Cheuk-Kwan Sun, Fan-Yen Lee and Hon-Kan Yip
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Olson LE, Bedja D, Alvey SJ, Cardounel AJ, Gabrielson KL and Reeves RH (2003) Protection from doxorubicin-induced cardiac toxicity in mice with a null allele of carbonyl


Footnotes

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Yung-Lung Chen and Sheng-Ying Chung are equal contributions.

Jiunn-Jye Sheu and Hon-Kan Yip are both correspondences.
Figure Legends

Figure 1. Doxorubicin induced DNA damage and carvedilol therapy upregulated the capacity of DNA repair

A) The protein expression of γ-H2AX progressively enhanced, whereas the protein expression of Ku-70 and phosphorlyated (p)-Akt were progressively attenuated after stepwise increased doxorubicin dosage (i.e., from 0, 20, 100 to 500 nM).  

B) The protein expressions of Ku-70 and p-Akt (i.e., two indicators of DNA repair) progressively enhanced as the dose of carvedilol increased (i.e., 0, 2, 5 to 10 µM).

Figure 2. In vitro study of protective effect of carvedilol on DNA damage Induced by doxorubicin

A) Demonstrating the dosage and timing of doxorubicin and carvedilol to be utilized in co-culture with H9C2 cells.  

B) The protein expressions of Ku-70, p-Akt and cardiac troponin I-interacting kinase (TNNI3K), three indicators of DNA-repair were notably progressively downregulated at two time interval points (i.e., at 24 h or extended to 48 h) in doxorubicin (100 nM) treatment, whereas these three parameters were stepwise upregulated in carvedilol (10 µM) treatment at two time interval points.

Figure 3. Immunofluorescent (IF) stain for identifying doxorubicin induced DNA damage that was reversed by carvedilol therapy

A to D) IF microscopic finding (400x) illustrated the CD90/XRCC1+ cells.  Red color indicated positively stained XRCC1, green color indicated positively stained CD90, positively double stain (i.e., red and green colors) indicated CD90/XRCC1+ cells.  

E) Analytical result of CD90/XRCC1+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001.  

F to I) IF microscopic finding (400x) illustrated the CD90/53BP1+ cells.  Red color indicated positively stained 53BP1, green color indicated positively stained CD90, positively double stain (i.e., red and green colors) indicated CD90/53BP1+ cells.
Analytical result of CD90/53BP1+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001. Scale bars in right lower corner represent 20 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Figure 4. Immunofluorescent (IF) stain for identifying doxorubicin augmented DNA damage and suppressed DNA repair that were reversed by carvedilol therapy

A to D) IF microscopic finding (400x) illustrated the γ-H2AX+ cells (red color). E) Analytical result of γ-H2AX+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001. F to I) IF microscopic finding (400x) illustrated the Ku-70+ cells (green color). J) Analytical result of Ku-70+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001. Scale bars in right lower corner represent 20 µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Figure 5. Doxorubicin treatment of dose-dependent augmentation of myocardial damage and alternation of heart weight and left ventricular function in living animals

A) Protein expression of Bax, * vs. other groups with different symbols (*, †, ‡), p<0.0001. B) Protein expression of cleaved caspase 3 (c-Casp 3), * vs. other groups with different symbols (*, †, ‡, §), p<0.0001. C) Protein expression of cleaved Poly (ADP-ribose) polymerase (c-PARP), * vs. other groups with different symbols (*, †, ‡), p<0.0001. D) The ratio of heart weight to tibial length, * vs. other groups with different symbols (*, †, ‡), p<0.0001. E) The analytical results of total heart weight, * vs. other groups with different symbols (*, †, ‡), p<0.001. F) The analytical results of left ventricular ejection fraction (LVEF), * vs. other groups with different symbols (*, †, ‡, §), p<0.0001. G) Analytic result of fibrosis area, * vs. other groups with different symbols (*, †, ‡, §), p<0.0001. H)
Analytic result of collagen deposition, * vs. other groups with different symbols (*, †, ‡, §), p<0.0001.  

I) Analytical result of 53BP1+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.001.  

J) Analytical result of γ-H2AX+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001.  

The lysate was loaded in a same SDS/polyacrylamide gel and then transferred to the PVDF membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane to assess the protein expressions of Bax, c-caspase 3 and c-PARP.  

All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=6 for each group).  

Symbols (*, †, ‡) indicate significance (at 0.05 level).  

Dox = doxorubicin.

**Figure 6. Carvedilol therapy against DOX-induced deterioration of heart function and enhancement of LV remodeling by day 35 after DOX (15 mg/ every-other-day for 2-Weeks) treatment**

A) Analytical results of left ventricular ejection fraction (LVEF), * vs. other groups with different symbols (*, †, ‡), p<0.001.  

B) Analytical results of left ventricular end-diastolic dimension (VLEDd), * vs. other groups with different symbols (*, †, ‡), p<0.001.  

C) Analytical results of left ventricular end-systolic dimension (LVESd), * vs. other groups with different symbols (*, †, ‡), p<0.001.  

All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=10 for each group).  

Symbols (*, †, ‡) indicate significance (at 0.05 level).  

Dox = doxorubicin; CAR = carvedilol.

**Figure 7. Protein expressions of apoptotic, NDA-damaged and mitochondria-damaged markers in LV myocardium by day 35 after DOX (15 mg/ every-other-day for 2-Weeks) treatment**

A) Protein expression of Bax, * vs. other groups with different symbols (*, †, ‡), p<0.001.  

B) Protein expression of cleaved caspase 3 (c-Csp 3), * vs. other groups with different symbols (*, †, ‡), p<0.0001.  

C) Protein expression of cleaved poly(ADP-ribose)
polymerase (c-PARP), * vs. other groups with different symbols (*, †, ‡), p<0.0001. D) Protein expression of γ-H2AX, * vs. other groups with different symbols (*, †, ‡), p<0.001.

E) Protein expression of cytosolic cytochrome C (cyt-Cyt C), * vs. other groups with different symbols (*, †, ‡), p<0.0001. F) Protein expression of mitochondrial cytochrome C (mit-Cyt C), * vs. other groups with different symbols (*, †, ‡), p<0.001. The lysate was loaded in a same SDS/polyacrylamide gel and then transferred to the PVDF membrane, followed by hybridization with different antibodies and with the use of β-tubulin as the control for each lane to assess the protein expressions of Bax, c-caspase 3 and c-PARP, and for each lane to assess the protein expressions of γ-H2AX and cyto-Cyt C, respectively. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=10 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Figure 8. Protein expressions of fibrotic, anti-fibrotic, and hypertrophic biomarkers in LV myocardium by day 35 after DOX (15 mg/ every-other-day for 2-Weeks) treatment

A) Protein expression of Samd3, * vs. other groups with different symbols (*, †, ‡), p<0.0001. B) Protein expression of transforming growth factor (TGF)-β, * vs. other groups with different symbols (*, †, ‡), p<0.001. C) Protein expression of Sam1/5, * vs. other groups with different symbols (*, †, ‡), p<0.0001. D) Protein expression of bone morphogenetic protein (BMP)-2, * vs. other groups with different symbols (*, †, ‡), p<0.0001. E) Protein expression of brain natriuretic peptide (BNP), * vs. other groups with different symbols (*, †, ‡), p<0.0001. F) Protein expression of beta-myosin heavy chain (β-MHC), * vs. other groups with different symbols (*, †, ‡), p<0.0001. G) Protein expression of alpha-myosin heavy chain (α-MHC), * vs. other groups with different symbols (*, †, ‡), p<0.001. The lysate was loaded in a same SDS/polyacrylamide gel and then transferred to the PVDF membrane, followed by hybridization with different antibodies and with the use of β-tubulin.
as the control for each lane to assess the protein expressions of p-Smad3, Smad1/5 and BMP-2. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=10 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Figure 9. Fibrosis and collagen deposition in LV myocardium by day 35 after DOX (15 mg/ every-other-day for 2-Weeks) treatment

A to C) Microscopic findings (100x) of Masson's Trichrome staining illustrating the fibrosis in LV myocardium among the three groups. D) Analytic result of fibrosis area, * vs. other groups with different symbols (*, †, ‡), p<0.0001. E-G) Microscopic findings (100x) of Sirius red staining illustrating the collagen deposition in LV myocardium among three groups. H) Analytic result of collagen deposition, * vs. other groups with different symbols (*, †, ‡, §), p<0.0001. Scale bars in right lower corner represent 100µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=10 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Figure 10. The expressions of DNA-damaged and DNA-repaired cells in LV myocardium by day 35 after DOX (15 mg/ every-other-day for 2-Weeks) treatment

A to C) IF microscopic findings (400x) illustrated the expression of γ-H2AX+ cells in LV myocardium (pink color). D) Analytical result of γ-H2AX+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001. Scale bars in right lower corner represent 20µm. E to G) IF microscopic findings (200x) illustrated the expression of 53BP1+ cells in LV myocardium (red color). H) Analytical result of 53BP1+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001. Scale bars in right lower corner represent 50µm. I to K) IF microscopic findings (400x) illustrated the expression of Ku-70+ cells in LV myocardium (green color). L) Analytical result of Ku-70+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.0001. Scale bars in right lower corner represent 20µm.
All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=10 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.

Figure 11. Expressions of cardiac stem cells and endothelial cells in LV Myocardium by day 35 after DOX (15 mg/ every-other-day for 2-Weeks) treatment

A to C) Microscopic findings (400x) of immunohistochemical (IHC) staining identifying the expression of Sca-1+ cells in LV myocardium (red arrows). D) Analytical result of Sca-1+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.001. Scale bars in right lower corner represent 20µm. E to G) Microscopic findings (400x) of IHC staining identifying the expression of Sca-1+ cells in LV myocardium (red arrows). H) Analytical result of Sca-1+ cells, * vs. other groups with different symbols (*, †, ‡), p<0.001. Scale bars in right lower corner represent 20µm. I to K) IF microscopic findings (200x) illustrated the expression of CD31+ cells in LV myocardium (yellow arrows). Scale bars in right lower corner represent 50µm. All statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison post hoc test (n=10 for each group). Symbols (*, †, ‡) indicate significance (at 0.05 level). Dox = doxorubicin; CAR = carvedilol.
Figure 1

(A)  

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<th>20</th>
<th>100</th>
<th>500</th>
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<td>γ-H2AX (15 kDa)</td>
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<tr>
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(B)  

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<th>10</th>
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</tr>
<tr>
<td>Tubulin (55 kDa)</td>
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</tbody>
</table>
Figure 2

(A)  
- + + - - -  Doxorubicin (100 nM, 24 h)  
- - - + + -  Doxorubicin (100 nM, 48 h)  
- - + - + +  Carvedilol (10 μM)  

(B)  
-  p-Akt (60 kDa)  
-  Akt (60 kDa)  
-  Ku-70 (70 kDa)  
-  TNNI3K (95 kDa)  
-  γ-H2AX (15 kDa)  
-  Tubulin (55 kDa)
Figure 6

(A) LVEF (%)

(B) LVEDd (mm)

(C) LVESd (mm)

Legend:
- SC
- Dox
- Dox+CAR

* p < 0.05
† p < 0.01