Long-acting PDE5 Inhibitor Tadalafil prevents early doxorubicin induced left ventricle diastolic dysfunction in juvenile mice: Potential role of cytoskeletal proteins

Journal: Canadian Journal of Physiology and Pharmacology

Manuscript ID: cjpp-2016-0551.R1

Manuscript Type: Article

Date Submitted by the Author: 08-Dec-2016

Complete List of Authors:
Nagiub, Mohamed; Virginia Commonwealth University School of Medicine, Internal Medicine
Filippone , Scott; Virginia Commonwealth University School of Medicine, Internal Medicine
Durrant, David; Virginia Commonwealth University School of Medicine, Internal Medicine
Das, Anindita; Virginia Commonwealth Univ. Medical Center, Internal Medicine
Kukreja, Rakesh; Virginia Commonwealth University School of Medicine, Internal Medicine

Keyword: cardiotoxicity, diastolic dysfunction, doxorubicin, Phosphodiesterase 5
Long-acting PDE5 Inhibitor Tadalafil prevents early doxorubicin induced left ventricle diastolic dysfunction in juvenile mice: Potential role of cytoskeletal proteins

Mohamed Nagiub¹.MD, Scott Filippone².MS, David Durrant².PhD, Anindita Das².PhD, Rakesh C. Kukreja². PhD.

¹Division of Pediatric Cardiology, Department of Pediatrics at Children’s Hospital of Richmond, Virginia Commonwealth University, Richmond, VA, USA.
²Pauley Heart Center, Division of Cardiology, Virginia Commonwealth University, Richmond, VA, USA

Corresponding Author:
Rakesh C. Kukreja PhD
Professor of Medicine, Physiology, Biochemistry and Emergency Medicine
Eric Lipman Distinguished Chair of Cardiology, Box 980281, Virginia Commonwealth University Medical Center, Richmond Virginia 23298-0281.Email: rakesh.kukreja@vcuhealth.org
Abstract:

The chemotherapeutic use of doxorubicin (Dox) is hindered due to the development of irreversible cardiotoxicity. Specifically, childhood cancer survivors are at greater risk of Dox-induced cardiovascular complications. Because of the potent cardioprotective effect of phosphodiesterase 5 (PDE5) inhibitors, we examined the effect of long-acting PDE5 inhibitor, Tadalafil (Tada) against Dox-cardiotoxicity in juvenile mice. C57BL/6 mice (6 weeks old) were treated with Dox (20 mg/kg, IV) and/or Tada (10 mg/kg/daily for 14 days; PO). Cardiac function was assessed by echocardiography following 5 and 10 weeks post treatment. The expression of cardiac proteins was examined by Western blot analysis. Dox treatment caused diastolic dysfunction in juvenile mice indicated by increasing the E/E’ (early diastolic myocardial velocity to early tissue Doppler velocity) ratio as compared to control at both 5 and 10 weeks post-treatment. Co-treatment of Tada and Dox preserved left ventricular (LV) diastolic function with reduction of E/E’. Dox treatment decreased the expression of SERCA2 and desmin in the LV, however, only desmin loss was prevented with Tada. Also, Dox treatment increased the expression of myosin heavy chain (MHCβ), which was reduced by Tada. We propose that Tada could be a promising new therapy for improving cardiac function in survivors of childhood cancer.

Key words: cardiotoxicity, diastolic dysfunction, doxorubicin, phosphodiesterase 5
Introduction:

Childhood cancer survivors are about 325,000 in US, a quarter of them are older than thirty years old (Mariotto et al. 2009). Cardiopulmonary diseases are the third-leading cause of death in survivors of childhood cancer, after the recurrence of primary cancer and the development of second cancers (Mertens et al. 2001). Chemotherapy induced cardiomyopathy is the first cause of cardiopulmonary problems among childhood cancer survivors, especially those treated with anthracycline (Lipshultz et al. 2013). Anthracycline (family name for doxorubicin) are among the most effective antineoplastic drugs and it is still used in nearly 60% of childhood cancer patients (Ries et al. 1999). Anthracycline shows direct cardiotoxicity in molecular, cellular and clinical studies (Gianni et al. 2008). Anthracycline-induced cardiotoxicity manifests as either early asymptomatic cardiac dysfunction or clinical heart failure due to dilated cardiomyopathy (Grenier and Lipshultz 1998). The diastolic dysfunction is due to calcium spark and/or impaired relaxation due to stiff tissue (Van Heerebeek et al. 2012). The impaired relaxation is secondary to one or more of the following mechanisms: cytoskeletal defects in Titin isomer (Katz and Zile 2006), desmin or nebulin (Leite-Moreira 2006)) and/or changes in extracellular matrix, metalloproteinase, increased expression of vimentin and collagen synthesis (Leite-Moreira 2006)) and/or endothelial dysfunction (Noireaud and Andriantsitohaina 2014). Several mechanisms of doxorubicin (Dox)-cardiotoxicity have been reported which include reactive oxygen species (ROS)/iron, cell death (necrosis/apoptosis/autophagy), changes in gene expression, activation of ubiquitin/proteasome system, and impaired cardiac repair by inhibiting progenitor cells.
and/or endothelial cell function (Shi et al. 2011). A number of protective strategies have been attempted to overcome Dox induced cardiotoxic effect, which include early detection to modify chemotherapeutic regimen and/or the use of protective agents. However, none of the known cardio protective drugs have shown superiority (Kalam and Marwick 2013).

Previous studies from our laboratory have shown that phosphodiesterase 5 (PDE5) inhibitors including sildenafil (Viagra) and Tadalafil (Cialis) attenuated cardiac dysfunction in Dox-induced cardiomyopathy in adult mice (Das et al. 2010; Das et al. 2005; Fisher et al. 2005; Fogli et al. 2004; Jin et al. 2013; Koka et al. 2010; Koka and Kukreja 2010; Kukreja et al. 2012). It was shown that treatment with these drugs before Dox administration inhibited cardiomyocytes apoptosis, preserved mitochondrial membrane potential ($\Delta \Psi_m$), myo-fibrillar integrity and prevented left ventricular systolic dysfunction as well as ST segment prolongation (Fisher et al. 2005). The cardio protective mechanisms included increase in cGMP, protein kinase G (PKG) activity, and manganese superoxide dismutase levels (Das et al. 2010). Interestingly, PDE5 inhibitors also enhanced cancer cell apoptosis through generation of ROS in metabolically deranged cancer cells with high lactate production with up regulation of Bad/Bax and suppression of Bcl2/Bcl-xL (Das et al. 2010).

Based on this background, we hypothesized that treatment with Tadalafil will prevent Dox-induced early LV diastolic dysfunction through alterations in the expression of cytoskeletal and contractile proteins in juvenile mice. Since the expression PDE5 is higher in the right ventricle (RV) than LV (Nagendran et al. 2007), that was explained secondary due to different embryological origin for each ventricle (Friedberg and
Redington 2014). Thus, we also examined the effect of Tadalafil on changes in the expression of cytoskeletal proteins in RV. We chose to use Tadalafil in this study because it is a long–acting PDE5 inhibitor with half-life of 17.5 hours. In clinical trials, tadalafil has been shown to be effective up to 36 hours after dosing, whereas the temporal effectiveness of sildenafil is 4 hours for the treatment of erectile dysfunction (Rosen and McKenna 2002).

Materials and Methods

Animal and experimental protocol:

The animal care and experiments were conducted under the Guide for the Care and Use of Laboratory Animals for Biomedical Research published by the National Institutes of Health (No. 85-23; revised 1996). The Institutional Animal Care and Use Committee of Virginia Commonwealth University approved the animal experimental protocols.

Mice (C57BL/6J) were purchased from Jackson Laboratories (Bar Harbor, ME) and randomly assigned into following four groups (n=10/group): 1. Control, (2) Dox (20 mg/kg, IV), (3) Tada (Tadalafil, Lilly, Indianapolis, IN, USA) (10 mg/kg/daily orally for 14 days), and (4) Tada + Dox. After 5 and 10 weeks of Dox treatment, cardiac function was monitored by Echocardiography. A sub-set of mice was anesthetized with pentobarbital sodium (70 mg/kg intra peritoneal) upon completion of Dox treatment. The hearts were quickly collected via thoracotomy, rinsed with saline, immediately frozen in liquid nitrogen, and stored at -80°C until protein analysis by Western Blot.

Assessment of LV function:
Transthoracic echocardiography (TTE) was performed using Vevo 770 imaging system (Visual Sonics, Inc., Toronto, Canada) under isoflurane inhalational anesthesia. B-mode images were obtained in the plane containing aortic and mitral valves, whereas M-mode images were obtained from the parasternal short-axis view at the level of papillary muscles and the apical four-chamber view. LV end-systolic (ESD), end-diastolic diameters (EDD) and fractional shortening (FS) were calculated by using VEVO Analysis software (version 2.2.3). Ejection fraction was not calculated as it depends up on volume quantification that assumed LV shape is bullet shaped in mice as human (Quinones et al. 1981; Nahar et al. 2000).

Diastolic function was assessed by measuring mitral inflow patterns (E, A, E/A) as well as lateral wall of LV tissue Doppler (e’ and E/e’) as described by Chen et al (Chen et al. 2012). E wave occurs during rapid filling phase in early diastole (passive process) and the A wave occurs in late diastole as a result of atrial contraction (active process). Sample volume is measured at the lateral side of mitral annulus to measure early e’ and late a’ diastolic mitral annulus velocities. The investigator performing echocardiography was blinded to the treatment status. These parameters were calculated at 5 weeks and 10 weeks from Dox treatment (Fig 1)

**Western Blot Analysis:**

Whole heart tissue samples were collected and right as well as left ventricles were dissected. The protein was extracted by homogenizing in a buffer containing 50-mmol/l potassium phosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2-mmol/L phenyl methyl sulfonyl fluoride, 5-mmol/L -glycerophosphate, 2 mmol/L NaF, 2 mmol/L Na₃VO₄, 10 mmol/L mercaptoethanol, 1 g/mL pepstatin, and 0.5 g/mL leupeptin, (pH 7.0). The
homogenate was centrifuged at 12,000g for 10 min under 4°C and the supernatants were recovered. Bradford protein assay was performed to measure protein concentration. Protein (50 µg) from each sample was separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membrane was incubated (overnight) with primary antibodies at a dilution of 1:1000 for each of the proteins: Myosin light chain (Cell Signaling, L57A3), Myosin heavy chain β (Santa Cruz, H300), actin (Cell signaling, β actin), desmin (Cell Signaling, desmin), vimentin (Cell Signaling, Vimentin), SERCA2 (Thermo Fischer, SERCA2), PDE5 (Cell Signaling), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (Sigma-Aldrich) as loading control. The membrane was washed and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution). The blots were developed using a chemiluminescent system (Amersham ECL Plus; GE Healthcare Bio-Sciences Pittsburgh, PA) and subsequently exposed to Kodak film. The optical densities of the protein bands were quantified using image J software.

Statistical analysis:
Statistical analysis was performed using Prism software version 7 (Graph Pad Software Inc., San Diego, CA). Data are presented as mean ±standard error (SEM). The difference between groups was analyzed by analysis of variance followed by Man Whitney test. Statistical differences were considered to be significant at $p < 0.05$.

Results:
Survival:
A total of 40 mice were used in this study (10 mice/group). The survival rate was 100% in control as well as Tada, Dox and Tada+Dox treated groups.

**Tadalafil preserves diastolic function in Dox-treated mice**

Figure 2 shows the representative M-mode images from control, Dox, Tada and Dox+Tada-treated mice. There was no significant difference in left ventricular diastolic diameter (LVDD), left ventricular systolic diameter (LVSD) or fractional shortening (LVFS) between Dox and/or Tada treatment groups after 5 and 10 weeks of treatment (n=10 mice/group, p>0.05).

After 5 weeks of Dox treatment, E’ wave (lateral mitral early tissue Doppler wave) was decreased (9.2 ± 3.3 mm/sec) as compared to control mice (13.1 ± 4.8 mm/sec, n=10, p<0.05). Also E/E’ (ratio between early mitral inflow and lateral mitral early tissue Doppler wave) was higher in Dox-treated (54.3± 20.2) versus the control mice (27.4±20.8, n=10, p<0.05, Fig 3). Interestingly, after 10 weeks of Dox treatment, E’ was further reduced (7.2 mm/sec ± 2.6) as compared to controls (13.7 ±2.8 mm/sec) (n=10, p<0.001 control vs Dox treated). Accordingly E/E’ ratio was increased in Dox-treated (81.2±9.7) versus the control mice (33.6 ± 9.7, n=10, p<0.005, Fig 3). After 5 weeks of cotreatment of Dox and Tada increased E’ wave (13.1 ± 4.9 mm/sec) as compared to Dox alone (9.2 ± 3.3 mm/sec, n=10, p>0.05). Consequently, E/E’ was reduced (although non-significant) following combination treatment with Tada and Dox (47.5±22.1) as compared to the mice treated with Dox only (54.3±20.2, n=10, p>0.05). At 10 weeks E’ also increased in Tada and Dox treated (14.7 ±8.7 mm/sec) versus Dox-treated mice (7.2 ± 2.6 mm/sec, n=10, p <0.05, Fig 3). Therefore, the E/E’ was
significantly reduced in Tadalafil and Dox-treated mice (40.3±30.4) as compared to Dox only (81.2±38.9, n=10, p<0.05, Fig 3).

**Expression of PDE5, Cytoskeletal and Contractile Proteins**

The baseline expression of desmin, SERCA2, and myosin light chain (MYL) was higher in LV than RV (n=3, p<0.05, Fig 4). The baseline expression of myosin heavy chain β (MHCβ), PDE5 and vimentin was higher in RV as compared to LV (n=6, p<0.05, Fig 4). Following treatment with Dox, SERCA2 was down regulated in both LV and RV (n=3, p<0.05, Fig 5 A, B). Desmin expression was decreased in LV (n=3, p<0.05) while remained unchanged in RV following Dox treatment (Fig. 5 A, B). Dox treatment caused increase in vimentin expression in both LV and RV (n=6, p<0.001) (Fig. 6A, B). Dox treatment had no effect on MYL3 in both the chambers (n=3, p>0.05 Fig 7 A, B)). MHCβ increased in RV (n=3, p<0.05) while remained unchanged in LV following Dox treatment (Fig. 7 A, B). Dox did not alter PDE5 in both the chambers (Fig. 8 A, B).

Treatment with Tada alone up-regulated SERCA2 only in the RV (n=3, p<0.001, Fig 5) while having no effect on other proteins in LV or RV (n=3-6, p>0.05). Combination treatment of Tada with Dox had differential effect in restoring the expression of proteins. Dox-induced MHCβ expression was prevented by co-treatment with Tada in RV (n=6, p<0.05), but not in LV (n=6, p>0.05; Fig. 7A, B); Desmin loss was prevented in LV (n=3, p<0.05) while no effect on RV; SERCA2 loss was prevented in RV (n=3, p<0.05) but had no effect in LV (n=3, p>0.05) (Fig. 5 A, B). Similarly, Tada prevented Vimentin increase in RV (n=6, p<0.001) but had no effect in LV (n=6, p>0.05)
(Fig. 6 A, B). Finally the combination treatment had no effect on MYL or PDE5 expression in both the ventricles ($n=3$, $p>0.05$) (Fig. 7 A, B and 8 A, B).

**Discussion:**

Almost 60% of children diagnosed with cancer receive Dox as part of their treatment protocol (Van Dalen et al. 2007) and a high percentage of cardiac dysfunction occurs in childhood cancer survivors’ following years or even decades after Dox chemotherapy (Lipshultz 2006). Thus, there is a great need to develop new and novel strategies to alleviate Dox-induced cardiotoxicity for children. In the present study, for the first time, we have shown that LV diastolic dysfunction was detectable following single intravenous administration of Dox (20 mg/kg) in juvenile mice as early as 5 weeks after treatment. Moreover, daily oral treatment with the long acting PDE5 inhibitor Tada (10 mg/kg) reversed diastolic dysfunction caused by Dox at 10 weeks of post treatment. Our results also showed that Dox treatment caused differential regulation of PDE5, contractile and cytoskeletal proteins in the LV and RV that either individually or in concert may have contributed to the early diastolic dysfunction in juvenile mice.

Dox cardiomyopathy in the clinical setting is commonly evaluated by measurement of EF or FS, either by nuclear methods (multi-gated radionuclide ventriculography or MUGA) or by echocardiography. However EF or FS are insensitive to detection of early Dox cardiomyopathy. Early detection of Dox-induced cardiotoxicity recommended by the new guidelines of American Society of Echocardiography is the measurement of early diastolic changes including LV strain, tissue Doppler, tricuspid/mitral annular plane excursion rather than EF or FS (Plana et al. 2014; Shankar
et al. 2008). Accordingly, E/E’ is an accurate method to predict LV filling pressure (Nagueh et al. 2009). Thus in the present study, we used the ratio between early mitral inflow velocity and lateral mitral early tissue Doppler wave (E/E’) for detection of diastolic dysfunction. It has been reported that Dox treated adult rats had significant reduction in radial strain as compared with control rats by 8 weeks of treatment (Migrino et al. 2008). These authors reported decreased FS by 12 weeks of treatment whereas our results showed preserved FS up to 10 weeks post treatment with diastolic dysfunction. These differences could be attributed to the treatment schedule of Dox and animal species i.e., adult rats versus juvenile mice used in our current experiments.

In the present study, we observed baseline expression of PDE5 that was higher in RV as compared to LV (Fig. 8), but it did not increase following Dox treatment. Previous studies have shown that PDE5 is up-regulated 2- to 6-fold in experimental mice and human heart disease (Nagendran et al. 2007). Ventricular PDE5 expression is increased in patients with advanced heart failure and contributes to adverse ventricular remodeling after myocardial infarction in mice (Tsai and Kass 2009). Chronic inhibition of PDE5 prevented and reversed cardiac hypertrophy (Takimoto et al. 2005). It has been shown that PDE5 cytosolic cGMP hydrolytic activity increases from 22% in non-failing heart to 43% in the failing heart (Vandeput et al. 2009), which potentially increases its influence and pharmacologic impact from its subsequent inhibition. Failure of Dox to increase PDE5 expression in the current study could be due to the age of these mice. Moreover, these hearts had not developed LV hypertrophy or advanced heart failure.
Our results showed that Dox treatment down regulated SERCA2 in LV that was not prevented by Tadalafil despite preserved systolic function. The main ionic efflux mechanisms in cardiac myocytes are the plasma membrane Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) and the Ca\(^{2+}\) ATPase (SERCA) pump on the SR. Diastolic calcium spark occurs due to persistence of calcium in the SR due to malfunction of SERCA2 or NCX resulting in sustained contraction during diastole (Periasamy and Janssen 2008). It has been suggested that there is no low absolute SERCA2 level that is linked to LV systolic failure. Moreover, SR calcium transport is not exclusively dependent on SERCA protein (Bers et al. 2003). In particular, the calcium transients from failed myocytes were significantly smaller and decayed more slowly than non-failing myocytes with different mechanisms as SERCA2 and NCX (Piacentino et al. 2003). In the present study, SERCA2 expression in RV was increased following Tadalafil treatment. It has been reported that treatment with sildenafil decreased pulmonary pressure with lower LV diastolic pressure (Xie et al. 2012). Sildenafil improved RV contractility with the up regulation of SERCA2 in pulmonary hypertension patients. Also, treatment with sildenafil restored SERCA2 in failing heart (Gong et al. 2013).

Desmin expression was decreased in LV while remained unchanged in RV following Dox treatment (Fig. 5). Tadalafil treatment prevented the reduction of desmin expression in the LV while having no effect on RV. Desmin is a major muscle specific intermediate filament (IF) protein that maintains muscle cyto-architecture and connecting contractile apparatus to sub sarcolemmal cytoskeleton, the nuclei and other organelles (Paulin and Li 2004). Increasing desmin abnormalities have been correlated with the progression of diastolic dysfunction. Moreover, desmin contributes to the development of
diastolic dysfunction (Pawlak et al. 2015) and acute Dox cardiotoxicity is associated with a reduction of desmin (Arola et al. 2000). We had previously shown that Dox treatment caused myofibrillar disarray, abnormal desmin distribution, lack of Z-line integrity, and abnormal cytoplasmic desmin aggregation. Treatment with PDE5 inhibitor, sildenafil prevented these abnormalities (Fisher et al. 2005). Thus, Tadalafil treatment appears to mirror these findings in the juvenile mice based on the current data showing prevention of desmin drop.

In the present study, Dox treatment caused increase in the expression of vimentin in both LV and RV that was prevented in RV ($n=6$, $p<0.001$) with Tadalafil treatment but not in the LV ($n=6$, $p>0.05$) (Fig. 6). Activation of vimentin via phosphorylation disassembles the spatial reorganization of the vimentin network, leading to remodeling of the contractile elements and substantially affecting the intracellular force transmission during the smooth muscle contraction (Li et al. 2006). Recent studies demonstrated that vimentin production is triggered by enhanced production of ROS via a p21-activated kinase mediated signaling pathway (Li et al. 2009). Thus it is likely the Dox-induced ROS generation caused increase in vimentin expression in the LV (Wang et al. 2007).

Our results showed that MHC\text{\textbeta} increased in RV ($n=3$, $p<0.05$) while it remained unchanged in LV following Dox treatment (Fig. 7). Increase in MHC\text{\textbeta} in RV could be either due to the direct effect of Dox or secondary to pulmonary hypertension. Pulmonary hypertension can be secondary to LV diastolic dysfunction or higher LV end diastolic pressure following Dox treatment (Hole et al. 2013). Dox treatment increased MHC\text{\textbeta} with relative reduction of MHC\text{\textalpha} which improved with exercise (Hydock et al. 2009). Also, it has been reported that right ventricular volume overload increased $\beta$ to $\alpha$.
MHC ratio (Bartelds et al. 2011). Other studies have shown that treatment with Dox down regulated MHCβ in LV with no significant effect in RV (Lencova-Popelova et al. 2014)]. Similarly, MHCα, troponin and desmin decreased with Dox treatment, which was prevented by treatment with Tadalafil through cGMP signaling (Jin et al. 2013)]. There was no effect of Dox treatment on MYL expression in LV and RV.

It has been suggested that RV function is correlated with LV function through RV-LV cross talk results (Pawlak et al. 2015) and alterations of RV pressure changes LV pressure volume relationship (Little 1984). Also, 20-40% of systolic pressure and volume outflow results from LV contarcarion (Santamore and Dell'Italia 1998). Our data shows that Dox treatment caused increase in RV MHCβ that was normalized with Tadalafil treatment. This could be due to normalization of RV diastolic function although it was not measured in the present study. It has been shown that decrease in MHC ratio (α to β) impairs diastolic as well as systolic function (Bartelds et al. 2011; Fitzsimons et al. 1998). Similarly, Dox induced up regulation of RV vimentin may be linked to the diastolic dysfunction and decreased level of vimentin with Tadalafil treatment could be associated with normalization of diastolic function (Willmer et al. 2016). Similarly, Dox induced down regulation of RV SERCA2 and it was prevented by Tadalafil could be associated with the changes in diastolic function (Periasamy and Janssen 2008) as summarized in Figure 9.

In summary, we have shown that Dox induces LV diastolic dysfunction in the juvenile mice as early as early as 5 and 10 weeks of treatment with preserved systolic function. Chronic treatment with Tada preserved LV diastolic function after 10 weeks possibly through differential expression/ restoration of cytoskeletal and contractile
proteins in the LV and RV of Dox treated juvenile mice. Further studies are required to understand the cellular and molecular mechanisms and the possible cross talk between LV and RV in causing Dox-induced LV diastolic dysfunction. We propose that Tadalafil could be potentially an important drug in preventing Dox cardiomyopathy and improving cardiac dysfunction in survivors of childhood cancer.

Authors Contributions:
All authors contributed equally to this work.

Acknowledgments:
This material is based upon work supported by the national institute of Health (NIH) HL51045, 79429 to RCK and American Heart Association (AHA) 0765273U pre-doctoral fellowship to DD, CCSA (UL1TR000058 from the National Center for Advancing Translational Sciences) and the CCTR (Center for Clinical and Translational Research) Endowment Fund to AD.

Conflict of interest:
None

References


and left ventricular dysfunction in a chronic model of doxorubicin cardiotoxicity. Circulation, 111(13): 1601-1610. DOI: 10.1161/01.CIR.0000160359.49478.C2


phosphodiesterase 5A prevents and reverses cardiac hypertroph. Nature, 11(2):214-222. DOI: 10.1038/nm1175


Figures legends

**Figure 1:** Outline of the experimental protocol.

**Figure 2:** Transthoracic echocardiography (TTE) assessment of the ventricular contractile dysfunction following treatment with Dox and/or Tadalafil. Representative echocardiographic tracings for each of the 4 experimental groups. A: Control, B: Doxorubicin, C: Tadalafil, D: Dox and Tadalafil. Bar diagrams showed averaged data of 5/10 weeks LVDD (E), 5/10 weeks LVSD (F) and fractional shortening for 5/10 weeks (G).

Abbreviations: Dox - doxorubicin, Tad- Tadalafil, D: Dox and Tadalafil. LVDD: left ventricular diastolic diameter, LVSD: left ventricular systolic diameter and FS: fractional shortening. Data is expressed as means ± SEM, n=10/group. In 5 weeks, \*p>0.05 for all systolic parameters.

**Figure 3:** TTE assessment of diastolic function following treatment with Dox and/or Tadalafil.

Representative echocardiographic tracings for each of the 4 experimental groups. A: Control, B doxorubicin, C: Tadalafil, D: Dox and Tadalafil. Bar diagrams showed averaged data of mitral E wave velocity (E), lateral E’ velocity (F), mitral E/A (G) and E/E’ ratio (H). Data is expressed as means ± SEM, n=10/group. In 5 weeks, Mitral E (p>0.05), Lateral E’ (\*p<0.05 versus Dox), and E/E’ (\*p<0.05 versus control); in 10 weeks, Lateral E’ (\"p<0.05 versus others) and E/E’ (\"p<0.05 versus others).
**Figure 4: Baseline differences in the expression of proteins between RV and LV.** Data is expressed as means ± SEM, n=3-6/group. *p<0.05 versus RV for desmin, SERCA2 and MYL3. *p<0.05 versus LV for MHCβ, PDE5 and Vimentin.

**Figure 5: Representative Western Blots and bar diagram showing expression of SERCA and Desmin in LV and RV following treatment with Dox and/or Tadalafil.** A. Representative immunoblots for Desmin and SERCA. B. Densitometric analysis of the ratios of Desmin to GAPDH and SERCA to GAPDH. *p<0.05 versus control and Dox/Tada for LV desmin. *p<0.05 versus control for LV SERCA2. Data is expressed as means ± SEM, n=3/group. *p<0.05 versus control and Dox+Tada for RV SERCA2. a p<0.01 versus others for RV SERCA2.

**Figure 6: Representative Western Blots and bar diagram showing expression of vimentin in LV and RV following treatment with Dox and/or Tadalafil.** A. Representative immunoblots for vimentin. B. Densitometric analysis of the ratios of vimentin to GAPDH. Data is expressed as means ± SEM, n=6/group. **p<0.01 versus control for LV vimentin. **p<0.01 versus others for RV Vimentin.

**Figure 7: Representative Western Blots and bar diagram showing expression of MHCβ and MYL in LV and RV following treatment with Dox and/or Tadalafil.** A. Representative immunoblots for MHCβ and MYL (n=3). B. Densitometric analysis of
the ratios of MHCβ to GAPDH (n=6/group) and MYL to GAPDH (n=3/group). Data is expressed as means ± SEM. *p<0.05 versus control and Dox/Tada for RV MHCβ.

**Figure 8**: Representative Western Blots and bar diagram showing expression of PDE5 in LV and RV following treatment with Dox and/or Tadalafil. A. Representative immunoblots for PDE5. B. Densitometric analysis of the ratios of PDE5 to GAPDH. Data is expressed as means ± SEM, n=6/group.

**Figure 9**: Proposed scheme of Dox induced left ventricular diastolic dysfunction and restoration by tadalafil in juvenile mice.
Figure 1

C57BL/6J
n = 10
6 weeks old

Doxorubicin 20 mg/kg IV for Dox and Dox+Tada groups

Tadalafil 10 mg/kg daily for 14 days

2 weeks
3 weeks
5 weeks

5 weeks post Dox:
Systolic function: LVDD, LVSD and FS
Diastolic function: mitral E, lat E', mitral E/A and E/E'

Sacrificed, dissected right from left ventricle.
Western blot: PDE5, MHC-β, MYL, SERCA2, Vimentin and desmin

10 weeks post Dox:
Systolic function: LVDD, LVSD and FS
Diastolic function: mitral E, lat E', mitral E/A and E/E'
Figure 2

A: Control, B: Dox, C: Tada, and D: Dox+Tada

https://mc06.manuscriptcentral.com/cjpp-pubs
Figure 4

n=3-6, * p<0.05 vs RV
p<0.05 vs LV

Protein/GAPDH

LV
RV

MHC
Desmin
SERCA2
MYL3
PDE5
Vimentin
Figure 5

A

LV

<table>
<thead>
<tr>
<th>Control</th>
<th>Dox</th>
<th>Tada</th>
<th>Dox + Tada</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Control" /></td>
<td><img src="image2" alt="Dox" /></td>
<td><img src="image3" alt="Tada" /></td>
<td><img src="image4" alt="Dox + Tada" /></td>
</tr>
</tbody>
</table>

RV

<table>
<thead>
<tr>
<th>Control</th>
<th>Dox</th>
<th>Tada</th>
<th>Dox + Tada</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5" alt="Control" /></td>
<td><img src="image6" alt="Dox" /></td>
<td><img src="image7" alt="Tada" /></td>
<td><img src="image8" alt="Dox + Tada" /></td>
</tr>
</tbody>
</table>

- **Desmin**
- **SERCA2**
- **GAPDH**

B

- **LV Desmin/GAPDH**
  - n=3, *p<0.05 vs Control and Dox + Tada
  - ![Graph](image9)

- **RV Desmin/GAPDH**
  - n=3, p>0.05
  - ![Graph](image10)

- **LV SERCA2/GAPDH**
  - n=3, *p<0.05 vs Control
  - ![Graph](image11)

- **RV SERCA2/GAPDH**
  - n=3, *p<0.05 versus control and Dox + Tada
  - ![Graph](image12)
  - p<0.01 versus others

https://mc06.manuscriptcentral.com/cjpp-pubs
Figure 6

A

LV

Control
Dox
Tada
Dox + Tada

RV

Control
Dox
Tada
Dox + Tada

Vimentin
GAPDH

B

n = 6, **p < 0.001 vs Control

LV Vimentin/GAPDH

Control
Dox
Tada
Dox + Tada

n = 6, *p < 0.01 vs Others

RV Vimentin/GAPDH

Control
Dox
Tada
Dox + Tada

https://mc06.manuscriptcentral.com/cjpp-pubs
Figure 7

A

<table>
<thead>
<tr>
<th></th>
<th>LV</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dox</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tada</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dox + Tada</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- MHCβ
- GAPDH
- MYL3
- GAPDH

B

- LV MHCβ/Actin: n=6, p>0.05
- RV MHCβ/Actin: n=6, *p<0.05 vs Control, **p<0.005 vs Dox + Tada
- LV MYL3/GAPDH: n=3
- RV MYL3/GAPDH: n=3,
Figure 8

A

LV

Control  Dox  Tada  Dox + Tada

RV

Control  Dox  Tada  Dox + Tada

n = 6, P > 0.05

B

LV PDE5/GAPDH

n = 6, P > 0.05

RV PDE5/GAPDH

n = 6, P > 0.05
Figure 9

Doxorubicin

↓ LV desmin

↑ RV MHCβ

↓ RV Vimentin

↑ RV SERCA2

Tadalafil

↑ RV MHCβ

↑ RV Vimentin

↓ RV SERCA2

↓ RV MHCβ

↓ RV Vimentin

↑ RV SERCA2

↑ LV desmin

↓ LV desmin

https://mc06.manuscriptcentral.com/cjpp-pubs