Cardiac response to doxorubicin and dexrazoxane in intact and ovariectomized young female rats at rest and after swim training

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Calvé A, Haddad R, Barama SN, Meilleur M, Sebag IA, Chalifour LE. Cardiac response to doxorubicin and dexrazoxane in intact and ovariectomized young female rats at rest and after swim training. Am J Physiol Heart Circ Physiol 302: H2048–H2057, 2012. First published March 23, 2012; doi:10.1152/ajpheart.01069.2011.—It is well recognized that increasing numbers of children now survive childhood cancer because of treatment improvements. However, almost two-thirds of childhood cancer survivors have at least one chronic illness and close to one-third had severe or life-threatening conditions when only in their mid-twenties (9, 44, 61). In addition, exercise testing of childhood cancer survivors has demonstrated a below-predicted exercise capacity (22, 63). The risk for cardiovascular disease is now a significant problem to adult survivors of childhood cancer. Specifically, doxorubicin (DOX) may be particularly harmful in young girls. The objective of this study was to characterize DOX damage and determine the ability of dexrazoxane (DEX) to reduce DOX-mediated cardiac damage in sedentary and swim-trained female rats. Female Sprague-Dawley rats were left intact or ovariectomized (OVX) at weaning then injected with DEX (60 mg/kg) before DOX (3 mg/kg), DOX alone, or PBS. Rats were separated into sedentary and swim cohorts. Body weight was reduced in DOX:DEX- but not PBS- or DOX-treated rats. Echocardiographic parameters were similar in sedentary rats. Swim training revealed greater concentric remodeling in DOX-treated rats and reduced fractional shortening in DOX:DEX-treated rats. Calsequestrin 2 was reduced with DOX and increased with DEX postswim. Sarco(endoplasmic reticulum Ca2+-ATPase 2a was reduced and calsequestrin 2 reduced further by swim training only in intact rats. OVX rats were heavier and developed eccentric remodeling post-swim with DOX and eccentric hypertrophy with DOX:DEX. Changes in SERCA2a and calsequestrin 2 expression were not observed. Ovariectomized DOX- and DOX:DEX-treated rats stopped growing during swim training. DEX coinjection did not relieve DOX-mediated cardiotoxicity in intact or hormone-deficient rats. DOX-mediated reductions in cardiac function, and expression of calcium homeostasis proteins were exacerbated by swim. DEX castration did not substantially relieve DOX-mediated cardiotoxicity in young female rats. Ovarian hormones reduce DOX-induced cardiotoxicity.

echocardiography; Sprague-Dawley rat; ovariectomy; sarco(endoplasmic reticulum Ca2+-ATPase 2a; calsequestrin 2

IT IS WELL RECOGNIZED THAT increasing numbers of children now survive childhood cancer because of treatment improvements. However, almost two-thirds of childhood cancer survivors have at least one chronic illness and close to one-third had severe or life-threatening conditions when only in their mid-twenties (9, 44, 61). In addition, exercise testing of childhood cancer survivors has demonstrated a below-predicted exercise capacity (22, 63). The risk for cardiovascular disease was found to be high and was attributed to anthracycline treatment years earlier (2, 27, 49, 54, 65). Anthracycline use, mostly doxorubicin (DOX), daunorubicin, and epirubicin, remains common, and it is estimated that about one-half of childhood cancer survivors will have received anthracyclines. Evidence suggests that young females may have a greater incidence of DOX-induced cardiotoxicity than young males (13, 26, 31, 32, 34, 58). Supporting this, DOX-exposed females showed greater cardiac function deficits than similarly treated males (18, 59). Thus activities of daily living and quality of life can be reduced in childhood cancer survivors as a result of their cancer treatment and females may be particularly at risk for the detrimental effects of DOX-induced cardiotoxicity.

DOX (Adriamycin; Adria)-mediated cardiac damage can be evident during, shortly after, or much later after its use (2, 11). Several mechanisms have been proposed for DOX cardiotoxicity including DOX-mediated increases in oxidative stress (2, 55). It has been proposed that increased oxidative stress reduce mitochondrial activity and lead to apoptosis (17, 29, 50). The mitochondria deficit has also been attributed to DOX-mediated reductions in the adenine nucleotide translocator protein leading to reduced mitochondrial respiration (45). DOX may also reduce cardiac function by altering calcium regulation via interactions with cardiac calsequestrin (CSQ2). CSQ2 may be particularly important for calcium signaling in heart because it is the principal calcium storage protein in the sarcoendoplasmic reticulum (SER) and helps to control the amount of calcium released for contraction (7). DOX was shown to form a complex with CSQ2 and reduce the ability of CSQ2 to bind calcium in vitro (48). In other experiments, rabbits injected with DOX showed SER-dependent contractile dysfunction linked to changes in sarco(endoplasmic reticulum Ca2+-ATPase 2a (SERCA2a) expression (46). SERCA2a re-questers calcium back to the SER allowing relaxation (15). Regulated expression of calcium homeostasis proteins, including CSQ2 and SERCA2α, is modified by sex and sex hormones (1, 12, 30, 56), suggesting a possible interaction between female sex and DOX sensitivity.

Dexrazoxane (DEX; ICRF-187, Zinecad; Pharmacia) [(+-)-1,2-bis (3,5-dioxopiperazinyl-1-yl) propane] (57) has a higher affinity for iron than DOX. Its use as a cardioprotectant is predicated on the finding that DEX reduces DOX-Fc complex formation, thereby lowering DOX-induced oxidative stress (28). DEX was shown to reduce the mitochondria toxicity of DOX in adult rats in vivo and rat cardiomyocytes in vitro (17, 29). DEX has reduced or prevented DOX-induced reductions in fractional shortening (FS) and left ventricular (LV) ejection fraction in children (6, 25, 53, 64). A recent study (32) identified greater DOX toxicity and greater benefit from DEX protection in female children (32).
Human (36, 43) and rodent (39) data suggest that female hormones can influence the extent of DOX-induced cardiac damage. However, the mechanism for greater DOX damage in female prepubertal children is unclear. Specifically, the ability of DEX to reduce DOX cardiotoxicities in the young has not been substantially evaluated. Previously, we (20) investigated the ability of DEX to reduce DOX cardiotoxicity in male and female day 10 neonate rats. We found that DEX did not reduce DOX-induced increases in apoptosis or oxidative stress and that DOX-induced cardiotoxicity was most evident in the young females. In this study, we probe an older age group closer to puberty. We analyze cardiac structure/function, expression of CSQ2 and SERCA2a, the ability to accommodate swim training of DOX-treated intact and ovariectomized rats, and the ability of DEX to reduce DOX-mediated cardiotoxicities.

**METHODS AND MATERIALS**

**Materials**

DOX and DEX were purchased from the Jewish General Hospital Pharmacy. Lactating Sprague-Dawley dams with female pups were purchased (Charles River Canada, St. Constant, QC, Canada). Primary antibodies SERCA2a (Santa Cruz Biotechnology, Santa Cruz, CA; N19 sc-8095, 1:1,000 dilution), cardiac CSQ2 (Abcam, Cambridge, MA; ab626662, 1:2,500 dilution), phospholamban (PLB; Thermo Scientific, Nepean, ON, Canada; 2D12 MA3–922, 1:10,000 dilution), and phospho-serine 16-specific PLB (pS16-PLB; Millipore, Temecula, CA; 07–052 1:1,000) were obtained commercially. Species-specific secondary antibodies complexed to horseradish peroxidase (1:10,000 to 1:20,000 dilution) and chemiluminescent detection kits were obtained from Pierce Chemical (Rockford, IL).

**Animal Manipulation**

All animal experimentation was performed after review and approval by the Lady Davis Institute Facility Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care, which are similar to those of the National Institutes of Health. A flow chart is shown in Fig. 1A.

**Intact and Ovariectomized Rat Cohorts**

On postnatal day (PND) 21 rats (n = 96) were randomized to remain intact (n = 48) or undergo ovariectomy (OVX; n = 48) on PND 23. Rats were anesthetized with isoflurane and ovariectomized using sterile techniques and approved surgical procedures. Carprofen (5 mg/kg) was given as analgesia. Successful ovarian excision in the OVX group was confirmed by a substantial decrease in uterine weight at euthanasia.

**Drug Treatments**

Rats (n = 16 per drug treatment) were injected intraperitoneally on PND 26 with either saline, DOX (3 mg/kg), or DOX:DEX (3 mg/kg DOX + 60 mg/kg DEX; 1:20 ratio). DOX was injected on the contralateral side 30 min after the DEX injection.

**Sedentary and Swim-Training Regimen**

Rats (n = 16 per drug treatment) were randomized into sedentary (SED; n = 8) or swim training (SW; n = 8) cohorts. Rats were swum in a tub, 90 x 60 x 25 cm, of 34°C water (3). Swim time was increased daily until 1 h of continuous swimming was achieved by the

![Fig. 1. A: schematic of experimental design. Rats (n = 8 per group) were weaned on day 21 and randomly assigned for ovariectomy (OVX) on day 23 or left intact. All rats were injected (INJ) intraperitoneally with PBS, DOX (3 mg/kg), or DOX:DEX (3 mg/kg DOX + 60 mg/kg DEX; 1:20 ratio). DOX was injected 30 min before the DOX injection on the contralateral side. Swim training began on day 30 and was completed on day 58. Echocardiographic acquisitions were collected 24 h after the last swim period. B and C: growth curve. All rats were weighed daily from weaning until euthanasia on day 59. Data are expressed as means ± SE. Significance: *P < 0.05, compared with PBS.](http://alphea.aph.org/)
end of week 1. Total swim time was 1 mo. Echocardiography and euthanasia were performed 24 h after the last swim period.

**Echocardiograph Monitoring of Cardiac Function**

Rats were anesthetized with 2% isoflurane and 1 l/min O2 and laid supine, and the chest was shaved. Echocardiography was performed using an i13L 14mHz linear transducer and a GE Vivid 7 ultrasonograph. At least three independent short axis acquisitions were collected at the midpapillary level. Data were analyzed offline from two-dimensional M modes. Measurements were taken from at least three consecutive beats using EchoPac software (GE Canada, Mississauga, ON, Canada). Intraventricular septum in diastole (IVSd), left ventricular posterior wall in diastole (LVPWd), and left ventricular internal diameter at diastole (LVIDd) and systole (LVIDs) were measured. Calculations were as follows: FS \[\frac{(LVIDd - LVIDs)}{LVIDd}\] × 100, left ventricular mass (LVmass) \[1.055(LVPWd + IVSd + LVIDd)^{\frac{3}{2}} - (LVIDd^3)\], and relative wall thickness (RWT) \[\frac{(LVPWd + IVSd)/LVIDd}\].

**Calcium Homeostasis Protein Expression**

 Portions of the LV were homogenized in RIPA buffer [50 mM Tris pH, 7.4, 150 mM NaCl, 0.5% Na deoxycholate, 0.1% NP10, 0.1% SDS, 1× complete proteinase inhibitor cocktail (Hoffman-La Roche, Laval, QC, Canada), 10 mM Na metabisulfite, 10 mM PMSF, 10 mM Na vanadate, and 1 mM okadaic acid], incubated on ice for 2 h, and then clarified by centrifugation. Total protein was measured using the Bradford assay against BSA. Ten to twenty micrograms of protein were electrophoresed through SDS-PAGE, the proteins electrophoretically transferred to Immobilon P (Millipore, Temecula, CA), and immunoblotting was performed by standard means. Several exposures from each membrane were collected onto X-ray film. The expression of each test protein is calculated relative to that of proteins stained by Coommassie on the same membrane.

**Statistical Analyses**

 Exposed films from immunoblots were scanned and the areas under the peaks quantitated using NIH Image 1.54. Statistical analyses used SigmaStat 3.1 and two- or three-way ANOVA as appropriate and Student-Newman-Keuls post hoc test. A P value of <0.05 was considered significant.

**RESULTS**

Weanling Sprague-Dawley female rats were chosen to model the impact of DOX and the ability of DEX to reverse DOX-induced cardiotoxicity in prepubertal female children. To determine if ovarian hormones reduced the impact of DOX cardiotoxicity, we performed OVX before normal puberty. Rats were swim trained to determine if DOX cardiotoxicity would be revealed if increased effort was demanded.

**Impact of Drugs on Growth**

To determine the time course of any DOX reduction in body weight (BW) gain of intact rats and to determine if DEX cotreatment could prevent growth reduction, we measured BW with time postinjection (Fig. 1, B and C). DOX:DEX-treated females had significantly reduced BW in intact rats and OVX rats and were lighter at euthanasia. All drug-treated groups in the OVX cohort were heavier than similarly treated intact rats. Thus DOX treatment had no impact on BW gain but the addition of DEX slowed BW gain.

Impact of Drugs on Echocardiographic Parameters

At baseline in sedentary rats. To determine if DOX altered cardiac structure, we performed echocardiography on SED rats (Table 1). No difference in any parameter measured was detected within the SED groups or within the drug-treated OVX-SED groups (Table 1). Thus DOX had no impact on resting LV structure/function in intact or hormone-deficient rats and coinjection with DEX had no effect.

To determine if ovarian hormone deficiency influenced the response to DOX or DOX:DEX, we compared intact and OVX rats. We found DOX:DEX-OVX-SED rats had lower FS when compared with intact DOX:DEX-SED-treated rats. However, all FS values are within the normal range for rats, suggesting no gross functional deficit and no impact of ovarian hormone deficiency on DOX sensitivity. Echocardiographic parameters in swim-trained rats. To determine if cardiac structure/function deficits not present when sedentary would be revealed with exercise, drug-treated rats were swim trained (Table 1). All rats completed the swim program.

**IMPACT OF DRUG TREATMENT WITHIN INTACT-SW OR OVX-SW RATS.** In intact-SW rats, RWT was reduced in DOX:DEX-SW (0.50 ± 0.03) rats when compared with that of the PBS-SW group (0.55 ± 0.02). In contrast, in OVX-SW rats, RWT was increased in DOX:DEX-OVX-SW (0.52 ± 0.03) rats over that detected in PBS-OVX-SW (0.45 ± 0.018) or DOX-OVX-SW (0.48 ± 0.01) rats. This suggests altered remodeling with swim training in the coinjected cohort in both intact and OVX rats.

**IMPACT OF OVARIAN HORMONE DEFICIENCY.** The impact of drug treatments was compared in intact-SW vs. OVX-SW rats. PBS-OVX-SW and DOX-OVX-SW rats had increased LVIDd and reduced RWT when compared with their respective intact PBS-SW or DOX-SW group. This suggests that hormone deficiency altered cardiac remodeling with swim but that DOX treatment had no additional impact. In contrast, no changes were detected when DOX:DEX-SW and DOX:DEX-OVX-SW rats were compared.

**Echocardiographic parameter remodeling as a consequence of swim training.** We compared echocardiographic parameters in age-matched sedentary and swim-trained rats to measure the impact of DOX and DOX:DEX on the ability to remodel. All intact swim-trained rats increased RWT without an increase in LVmass over that found in their sedentary cohorts. This suggests the development of concentric remodeling with swim training regardless of drug treatment. However, the increase in RWT in response to swim training was greater in the DOX-SW rats (0.41 ± 0.02 to 0.59 ± 0.03) than that of the PBS-SW (0.45 ± 0.02 to 0.55 ± 0.02) rats, suggesting some impairment. DOX:DEX-SW rats uniquely also had reduced FS.

When OVX-SED and OVX-SW rats were compared, the pattern in PBS-OVX-SW and DOX-OVX-SW rats, increased LVmass with no change in RWT, suggests eccentric hypertrophy development with swim training. In DOX:DEX-OVX-SW rats, the increased LVmass and increased RWT suggests concentric hypertrophy. Thus coinjected rats were more compromised and developed hypertrophy and functional deficits in intact and OVX rats.
Impact of Drugs on Physiological Parameters

At baseline in sedentary rats. To determine if DOX or DOX:DEX altered gross physiological parameters, we measured heart weight (HW), BW, and tibia length (TL) at euthanasia at 2 mo (Fig. 2). Within the intact cohort (Fig. 2A), PBS-SED and DOX-SED rats were similar. In contrast, BW parameters were reduced in DOX:DEX-SED rats, suggesting a leaner rat. Within the OVX-SED cohort (Fig. 2C), PBS-OVX rats were similar to PBS-SED and DOX-SED rats. BW parameters were reduced in DOX:DEX-OVX rats, suggesting a leaner rat. Within the OVX-SW cohort (Fig. 2D), BW parameters were reduced in DOX:DEX-OVX rats, suggesting a leaner rat. Within the intact cohort (Fig. 2A), PBS-SED and DOX-SED rats were similar. In contrast, BW parameters were reduced in DOX:DEX-SED rats, suggesting a leaner rat. Within the OVX-SED cohort (Fig. 2C), PBS-OVX rats were similar to PBS-SED and DOX-SED rats. BW parameters were reduced in DOX:DEX-OVX rats, suggesting a leaner rat. Within the OVX-SW cohort (Fig. 2D), BW parameters were reduced in DOX:DEX-OVX rats, suggesting a leaner rat.

Table 1. Echocardiographic parameters

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>DOX</th>
<th>DOX:DEX</th>
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<tr>
<td><strong>INT-SED</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVmass</td>
<td>616±51</td>
<td>651±21</td>
<td>651±25</td>
</tr>
<tr>
<td>RWT</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>IVSd</td>
<td>13.5±0.5</td>
<td>14.5±0.5</td>
<td>14.5±0.5</td>
</tr>
<tr>
<td>LVIDd</td>
<td>59.2±1.2</td>
<td>65.7±1.7</td>
<td>65.7±1.7</td>
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<tr>
<td>FS</td>
<td>49.4±1.7</td>
<td>53.1±1.7</td>
<td>53.1±1.7</td>
</tr>
<tr>
<td><strong>INT-SW</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LVmass</td>
<td>642±56</td>
<td>694±34</td>
<td>716±51</td>
</tr>
<tr>
<td>RWT</td>
<td>0.4±0.1</td>
<td>0.5±0.1</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>IVSd</td>
<td>15.7±0.8</td>
<td>15.7±0.8</td>
<td>15.7±0.8</td>
</tr>
<tr>
<td>LVIDd</td>
<td>57.3±2.1</td>
<td>63.6±1.9</td>
<td>63.6±1.9</td>
</tr>
<tr>
<td>FS</td>
<td>49.3±1.7</td>
<td>54.6±1.7</td>
<td>54.6±1.7</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Progeny rats (n = 8 per group) were intact (INT) or ovariectomized (OVX), and then separated into sedentary (SED) or swim-trained (SW) cohorts. Rats were swim trained for 4 wk. Rats were age-matched at the time of echocardiographic acquisition. Heart rate did not vary. DOX, doxorubicin; DEX, dexrazoxane. LV mass is calculated as 1.055 [(IVSd/LVPWd) x (LVIDd^3)] and is in mg; relative wall thickness (RWT) is in mm; left ventricular inner diameter in diastole (LVIDd) is in mm; fractional shortening (FS) is calculated as (LVIDd/LVIDs/LVIDd) x 100. Significance: *P < 0.05, compared with PBS; † P < 0.05, compared SED vs. SW; ‡ P < 0.05, compared intact vs. OVX.

Fig. 2. Physiological parameters at euthanasia. Rats (n = 8 per group) were euthanized on day 59. Body weight (BW), heart weight (HW), and tibia length (TL) were collected. Data are expressed as means ± SE. A: intact sedentary; B: intact swim trained; C: OVX sedentary; D: OVX swim trained. Solid bars are sedentary (SED) rats; hatched bars are swim-trained rats. Significance: *P < 0.05, compared with PBS; #P < 0.05, compared SED vs. SWIM; $P < 0.05, compared intact vs. OVX.
and DOX-OVX rats were similar but DOX:DEX-OVX-SED-treated rats were leaner than PBS-OVX-SED rats. All O VX rats were heavier than intact rats with the same drug treatment. Indexed HW was significantly increased in PBS-OVX-SED and DOX:DEX-OVX-SED groups compared with their respective intact groups implying hypertrophy. Thus DOX had no impact on HW or indexed HW but DEX addition reduced overall body size and indexed heart weight.

Physiological response to swim training. We compared the physiological parameters within either the intact or O VX group to determine if swim training revealed increased DOX cardiotoxicity and if this could be relieved by DEX (Fig. 2).

IMPACT OF DRUG TREATMENT WITHIN INTACT-SW OR O VX-SW RATS. In intact-SW rats (Fig. 2B), HW and indexed HW/TL were increased in DOX-SW and DOX:DEX-SW rats when compared with PBS-SW rats. This suggests greater cardiac hypertrophy development in the DOX treatment group that was not alleviated by DEX coinjection.

In O VX-SW rats (Fig. 2D), we found swim training reduced the BW in the DOX:DEX-OVX-SW compared with PBS-OVX-SW group. TL was significantly reduced in DOX-OVX-SW and DOX:DEX-OVX-SW cohorts, suggesting reduced growth in these swim trained rats.

IMPACT OF OVARIAN HORMONE DEFICIENCY. To determine if physiological remodeling was influenced by ovarian hormone deficiency, we compared drug treatment in intact-SW (Fig. 2B) vs. O VX-SW (Fig. 2D) rats. As expected, all parameters, except TL, remained greater in PBS-OVX-SW compared with intact PBS-SW rats. Thus growth was unaffected in PBS-SW rats regardless of hormone status but was significantly decreased in both DOX-OVX-SW- and DOX:DEX-OVX-SW-treated rats.

Physiological remodeling as a consequence of swim training. To determine how drug treatment altered physiological parameters changed by swim training in intact rats, we compared sedentary (Fig. 2A) and swim-trained (Fig. 2B) rats. As expected, BW/TL was reduced in all intact swim-trained rats regardless of drug treatment. Swim-trained PBS-treated rats did not develop cardiac hypertrophy but cardiac hypertrophy was induced in DOX-SW and DOX:DEX-SW rats. Thus DEX cotreatment did not relieve DOX-induced deficits in heart and body remodeling with swim training in intact rats.

To measure how drug treatment affected physiological remodeling with swim training in the absence of ovarian hormones, we compared the effect of drugs in O VX-SED (Fig. 2C) and O VX-SW (Fig. 2D) rats. Swim trained PBS-OVX rats had reduced BW but no cardiac hypertrophy. Swim trained DOX-OVX rats had reduced TL that was not relieved by DEX coinjection.

Impact of Drugs on SER Protein Expression

Calcium movement into the cytosol from the SER induces contraction, and its resequestration into the SER controls relaxation. DOX binds CSQ2 reducing its function and was shown to modulate SERCA2a expression. PLB and phosphorylation on serine 16 (pS16-PLB) control SERCA2a activity. We measured SERCA2a, CSQ2, PLB, and pS16-PLB protein expression to determine if DOX mediated changes in SER calcium homeostasis.

At baseline in sedentary rats. Expression in intact PBS-SED rats was artificially designated as 1 for all proteins. In intact rats (Fig. 2A), CSQ2 expression was reduced in DOX-SED rats. DEX-coinjected rats had increased CSQ2 expression, suggesting some counteraction of DOX cardiotoxicity in SED rats. In O VX rats (Fig. 2C), expression of all proteins was similar regardless of drug treatment.

When intact (Fig. 2A) and O VX (Fig. 2C) SED rats were compared, O VX, rats, regardless of treatment, had reduced SERCA2a and PLB but increased pS16-PLB compared with their intact counterparts. CSQ2 was reduced in coinjected O VX rats. Thus ovarian hormones are positive regulators for SERCA and PLB and are necessary for DOX-mediated reductions in CSQ2 and DEX induction of CSQ2.

SER expression changes as a result of swim training. We compared SER protein expression within the drug treatment to determine if drug treatment altered the molecular response to swim training (Fig. 3).

IMPACT OF DRUG TREATMENT WITHIN INTACT-SW AND O VX-SW RATS. Expression of all tested proteins was similar in intact PBS-SW (Fig. 3B) rats. This suggests no molecular remodeling of these proteins is necessary to accommodate swim training in control rats. In contrast, DOX-SW rats had reduced SERCA2a and CSQ2 expression that was increased in DOX:DEX-SW rats. This suggests that DOX treatment altered expression that was not reversed by DEX coinjection.

In O VX-SW rats (Fig. 3D), the expression of all the proteins was similar regardless of drug. This suggests no capability for DOX-mediated remodeling in hormone deficient SW rats.

IMPACT OF OVARIAN HORMONE DEFICIENCY. in intact-SW (Fig. 3B) vs. O VX-SW (Fig. 3D), PBS-OVX-SW rats had reduced PLB and increased pS16-PLB when compared with intact PBS-SW rats. DOX-OVX-SW rats had increased SERCA2a, CSQ2, and pS16-PLB over that of the intact DOX-SW cohort. Thus molecular changes were blunted in DOX-treated O VX rats and the addition of DEX had no additional impact.

Molecular remodeling as a consequence of swim training. We detected no change in expression when intact PBS-SED (Fig. 3A) and PBS-SW (Fig. 3B) were compared suggesting no molecular remodeling of these proteins was necessary to accommodate swim training. However, swim training resulted in reduced SERCA2a and CSQ2 expression in intact DOX-SW rats. In O VX rats (Fig. 3, A vs. D), PBS-OVX-SW rats had reduced PLB. We detected no change in the expression of any of the proteins as a consequence of swim training in DOX-O VX or DOX:DEX-OVX rats. The data suggest DOX-mediated changes in molecular remodeling that were reduced by DEX in intact rats and further, that ovarian hormones are necessary for DOX-mediated molecular remodeling.

DISCUSSION

DOX is a common component in the therapeutic treatment of acute lymphoblastic leukemia therapy of children, and most therapies are completed before puberty (51). Sprague-Dawley pups are normally weaned on day 23, and the presence of a vaginal opening on day 23 (range 30–34) generally indicates the onset of puberty. Thus we treated the rats at weaning to approximate the relative maturity window of children treated with DOX. Our study shows DOX toxicity in young rats occurred after a single DOX injection. DOX cardiotoxicity,
particularly in rats that underwent swim training, was revealed in altered echocardiographic and physiological indices and in an altered pattern of calcium homeostasis protein expression. We found DEX coinjection reduced BW gain and reduced cardiac function over that detected when DOX was delivered alone. However, DEX did alleviate DOX-mediated reductions in SERCA2 and CSQ2 expression. We conclude that DEX has a limited ability to reduce DOX cardiotoxicity, and for some parameters worsened DOX cardiotoxicity, in the young rat. We found that ovarian hormone deficiency increased DOX cardiotoxicity, suggesting a rationale for the enhanced DOX cardiotoxicity of the very young over that of older children and adolescents.

**DOX Toxicity in Intact Rats Is Not Alleviated by DEX Coinjection**

Our study shows that DOX alone did not reduce BW gain but that the combination of DOX:DEX contributed to a reduction in BW gain. DEX alone is not used clinically as a therapy, so we did not inject it as a standalone component. We cannot comment on any impact DEX-alone treatment might have had. Previously, we injected DOX and DOX:DEX treatment to PND10 neonate rats. In these very young rats, we found DOX-mediated reductions in BW gain, increased cardiac apoptosis, and increased reactive species generation that were not relieved by DEX (20). We also found reductions in vertebral bone growth and bone density (40–42). When DOX and DOX:DEX were given on PND 26 rats in the current study, TL was similar in sedentary intact rats regardless of treatment. This suggests that rat bone growth was unaffected by DOX in these older rats. Childhood survivors of cancer treated with combination therapies can have reduced standing and sitting heights (reviewed in Ref. 47), which have been attributed to the direct action of anticancer drugs on bone (14, 16, 23, 47). Our studies, using PND10 and PND26 rats, show that early DOX treatment slows BW gain but that the earliest treatment is more...
imparing because it also reduces bone growth. Further, our study shows that this effect is not reduced by DEX cotreatment in the young growing animal.

Echocardiography identified DEX-mediated reductions in DOX cardiotoxicity in nonstressed patients 5 yr after DOX treatment, and female children were shown to have a particular benefit (31, 32). We measured echocardiography 1 mo after DOX injection. We found that intact sedentary rats had similar echocardiographic parameters regardless of drug treatment. Our data suggest no effect of DOX or DOX:DEX at this level of DOX (3 mg/kg, injected once) and after this short time interval. Further, the data suggest that the addition of DEX had no effect on baseline echocardiograph parameters. This is in contrast to reductions in IVSd, FS, and RWT along with reduced survival detected in mature male rats treated with a single injection of 10 mg/kg DOX (19). We consider that the reduced effect in our rats is most likely a consequence of the lower dose of DOX we injected. DOX is known to persist in the heart (8, 60), bind to CSQ2, and reduce its calcium binding ability (48). The regulated flow of calcium into and out of the cytosol is recognized as essential for cardiac contraction and relaxation and thus overall cardiac function. Changes in calcium homeostasis protein expression, involving decreased SERCA2a expression and activity, are key features indicating heart failure (21). Regarding DOX-induced changes in protein expression, our data suggest reduced CSQ2 expression or increased CSQ2 destruction with no ability to normalize CSQ2 in DOX-treated rats. The increase in DOX:DEX rats can be seen as restorative. Overall, our data suggest similar SERCA2a activity in all drug-treated intact rats. A potential for reduced calcium storage via CSQ2 and thus possibly reduced calcium for release from the SER for contraction can be predicted in DOX-treated rats. The combined echocardiographic and expression data are consistent with the idea that cardiac function is for the most part maintained at near normal levels in the intact drug-treated heart. However, this normalcy occurs at the expense of larger changes in expression of calcium homeostasis proteins.

Our study shows that DOX-treated intact females successfully accommodated a moderate swim exercise program but that this is associated with greater cardiac remodeling and that DEX coadministration did not reduce the amount of remodeling necessary. All rats completed the swim training, and none had to be removed from the swim tank because of exhaustion. As expected, the BW and indexed BW were reduced, and HW and indexed HW were unchanged in PBS-treated swim trained arguing that the swim program was moderate. In contrast to lack of cardiac hypertrophy development in PBS-treated rats, cardiac hypertrophy developed in DOX- and DOX:DEX-treated rats. This indicates that the exercise was more taxing to DOX- and DOX:DEX-treated rats and sufficient to induce cardiac remodeling by this gross measure. The finding in the coinjected rats suggests that DEX did not reduce the requirement for cardiac enlargement.

Swimming is considered to be a highly dynamic and moderately static exercise (37). The response of prepubertal human athletes is distinctly different from adults, and the response of girls is not the same as boys. Echocardiography has identified increased LVIDd in prepubertal female competitive swimmers (62). We found swim training to induce concentric remodeling in all intact rats. However, the largest increase in RWT was detected in the DOX-treated rats, indicating that they remodel to a greater extent than other rats. DEX cotreatment allowed a similar increase in RWT as the PBS-treated rats, suggesting a reduction in DOX-mediated changes in cardiac structure. However, cardiac function, as measured by FS, was reduced in DOX:DEX-treated rats. FS is not generally considered to differ between athletes and nonathletes. Therefore, as expected, we found no difference in FS in the PBS swim-trained rats. We speculate that the decrease in FS in DOX: DEX-SW rats is perhaps an indicator that cardiac adaptation to swimming was beginning to fail in this group. It has to be noted that whereas no observed impact of DOX or DOX:DEX on echocardiographic indexes was evident in sedentary rats, DOX cardiotoxicity was revealed by swim training and was not relieved by DEX. Our results have characterized the echocardiograph changes induced by swim training in the young female rat. We suggest that the amount of swim exercise in this study was more taxing to DOX- and DOX:DEX-treated rats.

The effect of exercise on calcium homeostasis protein expression is unclear as few studies have used female rats (5, 10, 33), but changes in myocardial contraction are expected (24). No change in expression of SERCA2a or PLB but an increase in pS16-PLB was detected after swim training of adult male C57bl/6 mice (35). In adult male Wistar rats, high SERCA2a expression was correlated with high treadmill performance (52) but treadmill training has also led to no increase in SERCA2a (38). In contrast to all the above studies, we found no change in SERCA2a or its regulators, PLB or pS16-PLB, with swim training in PBS-treated rats. The differing results suggest either that no change in SER calcium homeostasis is necessary to accommodate moderate swim training or that the changes are related to differences in exercise type, sex, and age or rat strain. Regardless, swim-trained DOX-treated rats showed reductions in SERCA2a and CSQ2 expression that were normalized in the DOX:DEX-treated rats. The increase in the coinjected rats suggests some amelioration of DOX cardiotoxicity by DEX. These data suggest reduced calcium uptake and/or storage with swim training in the drug-treated rats. Furthermore, they suggest differences in the biochemical remodeling between DOX- and DOX:DEX-treated rats with swim. We found greater cardiac structure/function changes in the DOX-treated rats, along with greater changes in calcium homeostasis protein expression than detected in similarly treated PBS-treated mice. This argues that DOX treatment necessitated greater cardiac structure/function and molecular remodeling with swim training. The reduced cardiac function yet relatively normal molecular expression pattern in the DOX:DEX rats suggest that other proteins important to cardiac function are altered in the coinjected rats. Regardless of the nature of the protein changes, DEX did not fully reduce or alleviate DOX-mediated cardiac structure/function cardiotoxicities.

Ovarian Hormone Deficiency Increased DOX Cardiotoxicity

DOX treatment of children has led to cardiac function deficiencies when the children were past puberty and into young adulthood. We assessed the loss of ovarian hormones, and by implication female puberty, in mediating DOX cardiotoxicity and the ability of DEX to alleviate this toxicity. Similar to the intact rats, the DOX:DEX OVX-treated rats had...
reduced BW gain with time without a significant change in bone growth. Thus ovarian hormones do not influence DOX-mediated toxicity on BW gain or bone growth. At rest, and similar to our results with intact rats, there was no difference in any echocardiographic parameters in PBS- or DOX-treated rats. This suggests that baseline echocardiographic measures are not changed in the absence of ovarian hormones. Further, it suggests that the absence of female hormones did not influence DOX effects on baseline cardiac function. However, the reduction in FS and increase in LVmass when sedentary intact vs. OVX DOX:DEX-treated rats were compared suggests that ovarian hormones may reduce the impact of DOX:DEX treatment on cardiac function.

In rats ovariectomized when adult, SERCa2a expression was reduced and pS\textsuperscript{16}-PLB levels were unchanged, suggesting that female hormones are positive regulators of SERCa2a expression and implying that phosphorylation of PLB may be indirectly modulated to maintain SERCa2a activity (4). Consistent with this finding, we found reduced SERCa2a, CSQ, and PLB and increased pS\textsuperscript{16}-PLB in OVX PBS compared with intact PBS-treated rats. This supports the idea for some degree of ovarian hormone signaling as necessary for normal cardiac expression of these proteins. The increase in pS\textsuperscript{16}-PLB expression is likely secondary to the reduction in SERCa2a expression to maintain SERCa2a activity despite low SERCa2a protein. DOX and DOX:DEX treatment did not further affect expression of these proteins, suggesting that ovarian hormones are necessary for changes in their expression. Overall, our results show maintenance of cardiac function along with reductions in expression of key calcium homeostasis proteins in rats ovariectomized before puberty. Thus female hormones likely did not impact cardiac structure/function due to significant changes in protein expression. The lack of any significant further change with DOX or DOX:DEX implies that drug-treated hearts are incapable of further changes.

OVX impaired the ability to accommodate swim training and DEX treatment did not alleviate the effect of DOX-mediated reductions in cardiac function. Unlike the intact rats, all swim-trained DOX- and DOX:DEX-treated OVX rats reduced growth when swim trained. Thus the amount of swim training that was modest in the intact rats was more demanding to the DOX and DOX:DEX OVX rats. Further, no DEX mediation was noted. Unlike the intact rats that developed concentric remodeling with swim training, swim trained PBS-OVX and DOX-OVX rats developed eccentric remodeling. This remodeling progressed to eccentric hypertrophy in the DOX:DEX-OVX rats. Thus, OVX itself altered how cardiac structure was remodeled with swim training. The ability to accommodate swim training after OVX was not substantially worsened by DOX but was worsened by DOX:DEX treatment. There were no significant changes in SERCa2a, CSQ2, PLB, or pS\textsuperscript{16}-PLB in OVXd rats with swim training, suggesting that the capacity to change was lost. This is in contrast to the changes in SERCa2a, CSQ2, and PLB detected with swim exercise in the intact groups. Thus passage through female puberty would be expected to reduce DOX-mediated damage and response to cardiac stress. We conclude that the pathway to hypertrophy as a result of an increase in cardiac demand requires ovarian hormone signaling.

Our data clearly show that, directly or indirectly, DOX can damage the hearts of young prepubertal female rats. Moreover, our data show that this damage is not substantially relieved by DEX coinjection. It has been well established that the severity of damage induced by the same concentration of drug in the young is greater than that produced in the adult. Thus the minor myocardial injury of a single DOX injection led to greater subsequent myocardial impairment. This suggests that young cardiomyocytes are especially susceptible to damage. Our data suggest that DEX does not substantially protect these young cardiomyocytes from DOX damage. DOX itself is retained in cardiomyocytes and can be detected, albeit in much reduced amounts, weeks after the initial injection and well after it is undetectable in blood (8, 60). DOX thereby remains available for continued toxic action well past the initial insult. Its continued presence may permit it to have more harmful effects in the smaller and growing heart than in the larger and non-growing adult heart.

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REFERENCES


