Characterization of the Changes in Cardiac Structure and Function in Mice Treated With Anthracyclines Using Serial Cardiac Magnetic Resonance Imaging

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Background—Anthracyclines are cardiotoxic; however, there are limited data characterizing the serial changes in cardiac structure and function after anthracyclines. The aim of this study was to use cardiac magnetic resonance to characterize anthracycline-induced cardiotoxicity in mice.

Methods and Results—This was a longitudinal cardiac magnetic resonance and histological study of 45 wild-type male mice randomized to doxorubicin (n=30, 5 mg/kg of doxorubicin/week for 5 weeks) or placebo (n=15). A cardiac magnetic resonance was performed at baseline and at 5, 10, and 20 weeks after randomization. Measures of primary interest included left ventricular ejection fraction, myocardial edema (multiecho short-axis spin-echo acquisition), and myocardial fibrosis (Look-Locker gradient echo). In doxorubicin-treated mice versus placebo, there was an increase in myocardial edema at 5 weeks (T2 values of 32±4 versus 21±3 ms; P<0.05), followed by a reduction in left ventricular ejection fraction (54±6 versus 63±5%; P<0.05) and an increase in myocardial fibrosis (extracellular volume of 0.34±0.03 versus 0.27±0.03; P<0.05) at 10 weeks. There was a strong association between the early (5 weeks) increase in edema and the subacute (10 weeks) increase in fibrosis (r=0.90; P<0.001). Both the increase in edema and fibrosis predicted the late doxorubicin-induced mortality in mice (P<0.001).

Conclusions—Our data suggest that, in mice, anthracycline-induced cardiotoxicity is associated with an early increase in cardiac edema and a subsequent increase in myocardial fibrosis. The early increase in edema and subacute increase in fibrosis are strongly linked and are both predictive of late mortality. (Circ Cardiovasc Imaging. 2016;9:e003584. DOI: 10.1161/CIRCIMAGING.115.003584.)

Key Words: anthracyclines ◼ cardiotoxicity ◼ doxorubicin ◼ magnetic resonance imaging ◼ myocardium

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standard imaging technique for the detection and quantification of both edema and fibrosis; however, there are limited data validating the role of CMR in characterizing these pathological features of AIC.14 Therefore, the aim of this study was to test the role of the unique tissue characterization provided by CMR for the detection of AIC in a mouse model.

Methods
This was a longitudinal study of 45 wild-type C57BL/6 male mice (Jackson Laboratories, Bar Harbor, ME) aged 10 to 12 weeks randomized to doxorubicin (n=30) and placebo (n=15). The doxorubicin group received 5 mg/kg of doxorubicin/week for 5 weeks by subcutaneous pellet (Innovative Research of America, Sarasota, FL); in comparison, the control group received placebo at similar intervals. This dose and protocol was chosen based on laboratory experience and efforts to mimic clinic protocols.16

The experimental protocol is described in Figure 1. Key measures of interest included LV size, LVEF, myocardial edema, and the myocardial fibrosis measured at baseline, immediately after the first cycle of chemotherapy (within 48 hours), at 10 weeks (5 weeks after cessation of chemotherapy), and, in surviving animals, at 20 weeks (10 weeks after cessation of chemotherapy). Blood pressure was measured in all mice by tail-cuff manometry using a CODA-3 noninvasive blood pressure monitoring system (Kent Scientific, Torrington, CT) as previously described.17,19 All experiments were approved by the Institutional Animal Care and Use Committee.

Pathology
Subgroups of mice had pathological examination for confirmation of AIC, cardiac weight, and cardiac fibrosis at prespecified intervals as detailed in Figure 1.

Electron Microscopy
For pathological confirmation of AIC, we used the gold standard of electron microscopy.20 Representative myocardial sections from 5 mice per group at the 5-week time point were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde, and cacodylate buffer, at a pH of 7.4. From these, semithin sections were cut at 1 μm and stained with toluidine blue for light microscopic examination. Subsequently, ultrathin sections were cut from selected blocks at 80 nm, mounted on 200 mesh copper grids, treated with uranyl acetate and lead citrate, and examined in a JEOL 1010 transmission electron microscope (Tokyo, Japan) to obtain representative electron micrographic images of myocardial ultrastructure.

Histological Measurement of Cardiac Fibrosis
Hearts were fixed in formalin solution for histological analysis as previously described.17,18 In brief, sections of 5 μm in thickness were stained with Masson trichrome and viewed under polarized light using a 20x objective. Fifteen to 20 representative areas were chosen in each heart for collagen volume fraction analysis. The Spectrum Analysis algorithm package and ImageScope analysis software (version 9; Aperio Technologies, Inc, Vista, CA) were used. The fraction of collagen volume was calculated by counting the number of pixels occupied by the stained region and dividing this count by the number of pixels occupied by the entire section.

Cardiac Weight
Cardiac weight was determined by desiccation and comparison of the predesiccation versus postdesiccation weight as previously described.21

CMR Imaging
We performed serial CMR scans on a Bruker 9.4-T CMR imaging platform as previously described.13-15 In brief, for the CMR study, mice were anesthetized with isoflurane (induction 4% to 5%; maintenance 1% to 2% in oxygen from a precision vaporizer). For the CMR study, mice were placed in a special cradle, with electrocardiographic electrodes attached with tape to a front and back paw using electrode gel to optimize contact.

Conventional CMR Measurements
For LV volumes, mass, and LVEF calculation, the protocol was built on a self-gated (Bruker intragate, for cardiac phase–resolved, respiratory motion–compensated cine imaging) fast gradient-echo low-angle shot sequence with the following parameters as described: flip angle, 20°; repetition time (TR), 8.85 ms; TE, 2.36 ms; matrix, spatial resolution, 0.13×0.15 mm.13-15

Myocardial Edema by CMR
For assessment of myocardial edema, a multiecho short-axis spin-echo acquisition was performed with 5 spin-echo times (7.8, 15.6, 23.4, 31.2, 39, and 46.8 ms). The spin-echo acquisitions were both respiratory and electrocardiogram gated. The spin-echo parameters were as follows: 1 slice, slice thickness 1.0 mm, interslice distance 1.4 mm, matrix 128×128, spatial resolution 0.23×0.23 mm, and 2 averages. A repetition time of 200 to 400 ms was used, corresponding to triggering at every second or third cardiac cycles. Images were acquired precontrast. For processing of the T2 image series, the endocardial and epicardial borders of the LV were traced for all echo times. Because image quality was best on the mid-LV slice and the extracellular volume (ECV) is measured on the mid-LV slice, all T2 measurements were performed in this slice. To analyze the T2 signal, we used proprietary T2 mapping software. In brief, T2 was determined for each myocardial sector from a nonlinear least–squares fit of the measured echo amplitude as a function of echo time (TE) to an exponential function with constant offset (d); Mz(TE)=M0×exp(−TE/T2).

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Myocardial Fibrosis by CMR

The calculation of myocardial fibrosis by measurement of the ECV was based on precontrast and postcontrast T1 measurements as previously described in both humans and animals. In brief, gadolinium (0.2 mmol/kg) was diluted in saline solution in a 1:10 ratio and administered by multiple intraperitoneal injections. Myocardial T1 was measured in a mid-LV slice once before contrast and at least 4 times after contrast using a Look-Locker technique no earlier than 6 minutes after contrast administration. The T1 sequence was an electrocardiogram-gated Look-Locker sequence with an adiabatic nonslice-selective inversion pulse (hyperbolic secant inversion pulse) and the following parameters for the Look-Locker gradient-echo read outs: flip angle, 10°; TR, 2.2 ms; TE, 1.6 ms; in-plane resolution, 0.13×0.15 mm; slice thickness, 1 mm; repetition time per segment, 22 ms; and number of averages, 6 (precontrast) or 4 (postcontrast). Each Look-Locker acquisition was made ≥ 6 minutes after a subcutaneous injection of contrast. For 6 myocardial segments and the blood pool, signal intensity was plotted versus time after inversion. T1 values were obtained by nonlinear least-squares fitting of the curves of signal intensity versus time after inversion to an analytic expression for the magnitude of the signal measured during the inversion recovery. T1* was corrected for the radiofrequency pulse effects on the echo spacing, and the echo train length. The Marquardt–Levenberg algorithm was used for least-squares fitting, with M0, T2, and d, used as variable parameters, and best-fit estimates for these parameters were obtained by minimization of the residual sum of squares.

Statistical Analysis

Data are presented as mean±SD or median (range) if applicable. One of the key hypotheses was that the ECV at 10 weeks would be elevated compared with control mice. We planned on a ratio of 0.5 control mice to each doxorubicin-treated experimental mouse and 5 mice from each group to be euthanized at weeks 5 and 10 for histological analysis. In preliminary data, the ECV was normally distributed with a SD of 0.035. If the true difference in the experimental and control means was 0.045, we calculated that we would need 28 animals to survive to this time point. This estimation is using a probability (power) of 0.9. On the basis of previous work, we anticipated that at 10 weeks the mortality rate among doxorubicin-treated mice would be low, at 5% to 10%. Therefore, our initial sample size was 28 mice plus 10 mice compared with placebo, LV end-diastolic volume was 130±13 µL; LV mass was 99±15 versus 95±20 µg; and LVEF was 58±6% versus 62±4%.

T2 Measures by CMR

On CMR, there was an acute increase in T2 values at 5 weeks in doxorubicin-treated animals when compared with controls (32±4 versus 21±3 ms; P<0.001 for analysis of variance, and P=0.05 between groups; Table; Figure 3A). There was a close correlation between the native T1 values and the change in water weight (r=0.79; P=0.007; Figure 3B).

Native T1 Measures by CMR

On CMR, there was an acute increase in native T1 values at 5 weeks in doxorubicin-treated animals when compared with controls (1448±121 versus 1302±152 ms; P=0.03 for analysis of variance, and P<0.05 between groups; Table). There was a correlation between the native T1 values and the change in water weight (r=0.71; P=0.02; Figure 4) and a correlation between native T1 and T2 (r=0.33; P<0.01; Figure 5).

Myocardial Fibrosis by CMR

There was no change in ECV using the Look-Locker sequence either from baseline or between doxorubicin-treated animals and controls at 5 weeks (0.27±0.02 versus 0.26±0.03; Figure 6A). There was no difference in the percentage of fibrosis between doxorubicin- and placebo-treated animals at 5 weeks (2.9±0.2% versus 3.0±0.25%; P=0.45; Figure 6B).

10-Week Time Point

Conventional CMR Measures

At the 10-week time point, doxorubicin-treated animals had an increased LV end-diastolic volume (142±12 versus 128±11 µL; P<0.05) and a decreased LVEF (54±6% compared with 63±5%; P<0.05) in comparison to saline-injected mice. There was no statistical difference between doxorubicin- and saline-treated mice in terms of LV mass (93±13 versus 103±16 µg; Table).

Native T1 Measures by CMR

There was no difference between native T1 values at 10 weeks in doxorubicin-treated animals when compared with controls (1384±155 versus 1311±128 ms; P>0.05 between groups; Table).

T2 Measures by CMR

There was no difference in T2 values between doxorubicin- and placebo-treated groups at 10 weeks (22±3 versus 22±3 ms; Figure 3A).

Myocardial Fibrosis by CMR

On CMR, there was a subacute increase in ECV from baseline between doxorubicin-treated animals and saline-treated controls.
Characterization of AIC in Mice by CMR

at 10 weeks (0.34±0.03 versus 0.27±0.03; \( P<0.001 \) for analysis of variance, and \( P<0.05 \) between groups; Figure 6A). Fibrosis on CMR correlated strongly with the percentage of fibrosis on histology (\( r=0.93; \ P<0.001 \); Figure 6C). There was also a strong association between increase in fibrosis at 10 weeks and early increase in edema at 5 weeks (\( r=0.90; \ P<0.001 \); Figure 6D).

Mortality

Of the initial 30 mice randomized to the doxorubicin group, 10 were euthanized at prespecified time intervals for histology. Of the remaining doxorubicin-treated mice (n=20), none had died by the 5- or 10-week time points. By the 20-week time point, 15 of the 20 remaining doxorubicin-treated mice had died. From the control group, 10 mice were euthanized at prespecified intervals for histology. From the remaining 5 mice, none had died at the 20-week time interval. Figure 7 shows Kaplan–Meier curves with event rates for mortality related to native T1 (Figure 7A), T2/edema (Figure 7B), CMR-measured fibrosis/ECV (Figure 7C), and LVEF (Figure 7D). Both T2 and ECV conferred statistical difference to survival. Although LVEF was reduced at 10 weeks after chemotherapy, there was no difference in survival between groups separated by the median LVEF at 10 weeks.

Table. Cardiac Magnetic Resonance and Physiological Variables in Mice Treated With DOX or Placebo Over Time

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR, per min</th>
<th>MAP, mm Hg</th>
<th>LVEDV, µL</th>
<th>LV Mass, µg</th>
<th>LV Mass Index, µg/gm</th>
<th>LVEF, %</th>
<th>T2, ms</th>
<th>Native T1, ms</th>
<th>ECV</th>
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<tbody>
<tr>
<td>Baseline</td>
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<tr>
<td>Placebo</td>
<td>488±25</td>
<td>87±10</td>
<td>110±10</td>
<td>89±12</td>
<td>3.3±0.5</td>
<td>64±3</td>
<td>22±3</td>
<td>1296±158</td>
<td>0.25±0.03</td>
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<tr>
<td>DOX</td>
<td>490±25</td>
<td>89±9</td>
<td>114±9</td>
<td>89±15</td>
<td>3.3±0.5</td>
<td>63±4</td>
<td>22±3</td>
<td>1318±152</td>
<td>0.25±0.02</td>
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<td>5 wk</td>
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<tr>
<td>Placebo</td>
<td>498±20</td>
<td>90±9</td>
<td>122±13</td>
<td>95±20</td>
<td>2.9±0.8</td>
<td>62±4</td>
<td>21±3</td>
<td>1302±152</td>
<td>0.26±0.03</td>
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<tr>
<td>DOX</td>
<td>510±24</td>
<td>80±6*</td>
<td>130±13</td>
<td>99±15</td>
<td>3.6±0.7</td>
<td>58±6</td>
<td>32±4*</td>
<td>1448±121*</td>
<td>0.27±0.03</td>
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<td>10 wk</td>
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<tr>
<td>Placebo</td>
<td>500±17</td>
<td>92±8</td>
<td>128±11</td>
<td>103±16</td>
<td>2.9±6</td>
<td>63±5</td>
<td>22±3</td>
<td>1311±155</td>
<td>0.27±0.03</td>
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<tr>
<td>DOX</td>
<td>515±21</td>
<td>83±9</td>
<td>142±12*</td>
<td>93±13</td>
<td>3.0±0.6</td>
<td>54±6*</td>
<td>22±3</td>
<td>1384±128</td>
<td>0.34±0.03*</td>
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<td>20 wk</td>
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<tr>
<td>Placebo</td>
<td>511±16</td>
<td>97±8</td>
<td>132±11</td>
<td>111±18</td>
<td>2.8±0.5</td>
<td>63±4</td>
<td>21±3</td>
<td>1329±134</td>
<td>0.28±0.03</td>
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<tr>
<td>DOX</td>
<td>535±19*</td>
<td>81±6</td>
<td>161±15*</td>
<td>85±10</td>
<td>2.3±0.4</td>
<td>38±6*</td>
<td>21±3</td>
<td>1425±144</td>
<td>0.41±0.06*</td>
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*\( P<0.05 \) for comparison of DOX vs placebo.

Comparison of repeated measures was performed using an ANOVA (analysis of variance) and, if significant, the post hoc comparison was made using Tukey comparison test. DOX indicates doxorubicin; ECV, extracellular volume; HR, heart rate; LV, left ventricle; LVEDV, left ventricular end-diastolic volume; LVEF, left ventricular ejection fraction; and MAP, mean arterial pressure.
Discussion

The aim of this study was to test whether CMR could provide a longitudinal characterization of the pathological changes that have been described in AIC. We found that anthracyclines were associated with acute cardiac edema and subacute myocardial fibrosis, that the extent of edema and fibrosis was related, and that the extent of the early edema and the subacute fibrosis predicted the late doxorubicin-induced animal mortality in mice.

The presence of edema in our mouse model is consistent with the findings in cross-sectional pathological studies among patients administered anthracyclines.11,14,23–25 However, because of the invasive nature of cardiac biopsies, longitudinal clinical histological studies are limited. Therefore, a noninvasive method for characterizing the time course of cardiac edema in AIC may be of use. CMR is the gold standard imaging technique for the detection and quantification of myocardial edema.26–28 T2-weighted CMR sequences are sensitive to changes in myocardial water content.26–28 In this study, we measured cardiac edema within 48 hours of chemotherapy and found that there was an acute increase in cardiac edema in mice treated with anthracyclines; the increase was transient and had resolved at the next imaging time point. Similar increases in signal intensity suggestive of edema have been noted in clinical studies performed acutely after anthracyclines, and the acute change in edema has been correlated with the subsequent reduction in LVEF.29

Similar to edema, pathological clinical studies have consistently documented the presence of myocardial fibrosis in patients treated with anthracyclines.11,24,30,31 CMR is also the gold standard imaging technique for the measurement of myocardial fibrosis. There are 2 CMR methods for the detection of fibrosis, namely late gadolinium enhancement and T1 quantification or mapping, either alone or combined with the use of an extracellular contrast agent to generate an ECV.17–19,32–35 Although the late gadolinium enhancement is ideally suited for the detection and quantification of replacement myocardial fibrosis such as that which occurs with a myocardial infarct,36 the ECV and T1 measurements are ideally suited for the detection and qualification of the diffuse myocardial fibrosis such as that which occurs in AIC.17–19,22 In retrospective clinical studies, we and others have found that late gadolinium enhancement was an infrequent finding among patients treated with anthracyclines.37,38 In contrast, diffuse fibrosis by ECV was increased.22 In this study, we extend these findings and report that there was a subacute increase in the ECV at 10 weeks after starting anthracyclines in mice, that there was an association between the increase in ECV by CMR and the increase in histological myocardial fibrosis, that there was an association between the acute increase in edema and the subacute increase in the ECV, and that both edema and fibrosis predicted the late doxorubicin-induced mortality in mice.

We also tested the role of conventional measurement of cardiac structure and function after anthracyclines in mice and specifically the role of serial measurement of LVEF. CMR is a robust imaging technique for the reliable and reproducible measurement of LVEF,40 and cardiac surveillance with serial measurement of LVEF is recommended among patients treated with anthracyclines.41 Both baseline LVEF and the reduction of LVEF after anthracyclines therapy have been reported to predict clinical events.42–44 However, although measurement of

Figure 3. A, Measurement of cardiac edema using T2-weighted imaging between placebo-treated control mice and doxorubicin (DOX)-treated mice at baseline, 5, 10, and 20 wk showing an increase in cardiac edema in DOX-treated mice at 5 wk (*P<0.001 for analysis of variance, *P<0.05 for between group comparisons); (B) correlation between the cardiac magnetic resonance measurement of cardiac edema and cardiac weight.

Figure 4. Comparison of native T1 to cardiac water content revealing a positive correlation between native T1 values and the change in water weight.

Figure 5. Comparison of native T1 and T2 values showing a positive correlation between native T1 and T2.
LVEF is readily available and is a robust marker of outcomes in large populations, it has limitations in the monitoring of AIC. Among patients treated with anthracyclines, the LVEF is usually normal despite pathological evidence of extensive myocyte injury. A decline in LVEF is a late manifestation of AIC. Also, once decreased, the LVEF is minimally reversible, and a decrease in LVEF likely represents extensive myocardial injury beyond the ability of the heart to compensate. In support of this, we found that the LVEF was unchanged acutely after anthracyclines despite CMR evidence of edema and histological evidence of AIC. The LVEF did decrease subacutely after anthracyclines at the 10-week time point, which corresponded to the time at which subacute fibrosis was detected. However, we found no relationship between the reduction in LVEF subacutely after anthracyclines and the late animal mortality. In contrast, imaging characteristics focused on the histopathologic changes in the myocardium, edema and fibrosis, were both predictive of the late animal mortality.

This study should be interpreted within the context of the study design and has limitations that merit discussion. There is significant variability in the quantity, method of administration, and dosing schedule among animal studies testing the effect of anthracyclines on cardiac structure and function in mice. Therefore, these data are only relevant to this model and to mice;
however, we think that this study should form part of the basis of testing in a clinical study the role of the unique tissue character-
ization provided by CMR in tracking the histological changes consistent with AIC. Also, the amount of statistical testing used in our study (including multiple variables and time points) pro-
duced an error rate >0.05. Yet, using a Bonferroni adjustment would have been unlikely to change the interpretation of results for highly significant findings (e.g., P < 0.001).

In conclusion, these data suggest that, in mice, anthracy-
clines cause an acute increase in cardiac edema, a subsequent
increase in myocardial fibrosis, and that the acute edema and
subacute fibrosis are related and both predictive of the late
doxorubicin-induced animal mortality. Further research is
necessary to fully understand the stepwise pathogenesis of
AIC in clinical studies and test the role of edema and fibrosis
in the characterization of AIC in patients.

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Disclosures
None.

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